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LASER BIOEFFECTS: DIFFERENTIAL PROTEIN EXPRESSION OF CULTURED HUMAN MELANOCYTES TREATED WITH 532 nm PICOSECOND PULSE LASER-LIGHT

by

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A Thesis submitted to the Graduate Faculty of the

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Date

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Many thanks go to Lieutenant Colonel John W. Obringer, USAF (Ret.) for giving me the means to see the world through a genetic lens, and introducing me to the tools to investigate it. Organisms are indeed "competing bags of DNA!"

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Lykins, Daniel Robert (M.B.S, Biology)

Laser Bioeffects: Differential Protein Expression of Cultured Human Melanocytes Treated With 532 nm Picosecond Pulse Laser-Light.

Thesis directed by Assistant Professor Karen Newell

The use of laser light for targeting devices and weapons has dramatically increased the likelihood that personnel will be exposed to laser energy during military operations. The increased potential for exposure of humans to lasers highlights the need to understand laser-tissue interactions at the most basic cell and molecular levels. Current ultrashort pulse laser safety standards are based on a minimal visible lesion (MVL), i.e. histological, damage endpoint in the Rhesus monkey model (Shaver, 2001). A human model for assessing laser-light damage to tissue at the cell and molecular level is desirable for scientific, political and fiduciary reasons. This research assesses the effects of sublethal pulsed laser-light insult to a human melanocyte (skin) cell line. Proteomic inquiry into the cellular effect of laser insult was accomplished via parallel sample analysis with over 850 monoclonal antibodies in order to screen for changes in the levels of protein expression. In short, the data indicated dramatically increased molecular debridement, decreased apoptotic signaling and cell cycling and other cellular phenomena sufficient to propose a stress response and recovery paradigm.

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LASER BIOEFFECTS: DIFFERENTIAL PROTEIN EXPRESSION OF CULTURED HUMAN MELANOCYTES TREATED WITH 532 nm PICOSECOND PULSE LASER-LIGHT.

Daniel R. Lykins, Department of Biology, University of Colorado, Colorado Springs, in conjunction with John W. Obringer Ph.D., and Martin D. Johnson, Laser and Optic Research Center, Department of Physics, US Air Force Academy, Colorado Springs, Colorado.

Abstract

The use of laser light for targeting devices and industry has dramatically increased the likelihood that personnel will be exposed to laser energy during military and commercial operations. The increased potential for exposure of humans to lasers highlights the need to understand laser-tissue interactions at the most basic cell and molecular levels. Current ultrashort pulse laser safety standards are based on a minimal visible lesion (MVL), i.e. histological, damage endpoint in the Rhesus monkey model (Shaver, 2001). A human model for assessing laser-light damage to tissue at the cell and molecular level is desirable for scientific, political and fiduciary reasons. This research assesses the effects of sublethal pulsed laser-light treatment to a human melanocyte (skin) cell line. Proteomic inquiry into the cellular effect of laser treatment was accomplished via parallel sample analysis with over 850 monoclonal antibodies in order to screen for changes in the levels of protein expression. In short, the data indicated dramatically increased molecular debridement, decreased apoptotic signaling and cell cycling and other cellular phenomena sufficient to propose a stress response and recovery paradigm.

CHAPTER I

INTRODUCTION

[1]The use of laser light for targeting devices and weapons has dramatically increased the likelihood that personnel will be exposed to laser energy during military operations. Also, expanded medical, research, and industrial laser use may lead to excessive risk of exposure of researchers and technicians and during commercial applications. The increased potential for exposure of humans to lasers, especially sub-nanosecond laser pulses in the visible and near-infrared regions of the spectrum (Rockwell, 1999), highlight the need for scientifically based safety standards for laser exposure at the ultra short pulse lengths. The great peak powers achieved at ultrashort pulse lengths suggests that the current standards may not be appropriate to protect personnel. Further, the nature and relative importance of the biophysical mechanisms of photon-tissue interaction at such pulse widths and irradiances are not understood at the fundamental cell and molecular level. Current ultrashort pulse laser safety standards are based on a minimal visible lesion (MVL), i.e. histological, damage endpoint in the Rhesus monkey model (Shaver, 2001). A human *in vitro* model for assessing laser-light damage to tissue at the cell and molecular level is desirable for scientific, political and fiduciary reasons. This research assesses the effects of sublethal pulsed laser-light treatment to a human melanocyte cell line using BD Transduction Laboratories' BD PowerBlot™ Western Array screening service with antibodies for 859 known human proteins involved in cell signaling, apoptosis, cell adhesion, kinase and GTPase activity

[1]Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the United States Air Force.

as well as several other functions. Some of these functions have been shown to be important in biological processes that could lead to important biological sequelae such as loss of function, cancer or cell death. The results of Western array immuno-screening analysis of lased human melanocytes are discussed. This research was done in an effort gain basic scientific insight into the physiological state of cells at the level of protein expression after leaser exposure. Of specific interest is the amount and type of damage/perturbation cells undergo due to laser irradiation.

Statement of Purpose

The purpose of this research is to investigate the molecular bioeffects of high energy, ultrashort pulse laser-tissue interaction in skin cells using a tissue culture-based approach. An additional purpose is to provide an *in vitro* technology and database for US Air Force and other damage modeling efforts. The use of a protein expression benchmark allows indirect analysis of the up-regulation and down-regulation of specific genes, i.e. the transcriptional response, by examining the translational response (differential protein expression via immunologic screening of Western blots) of correlated experimental and control samples. This is expected to greatly improve the basic understanding of the cellular events and regulatory mechanisms governing the post sub-acute response of laser-exposed skin tissues.

Significance of the Study

Due to the proliferation of military and industrial lasers there is an increased the likelihood that personnel will be exposed to laser irradiation. Consequently, there is an increased need for scientifically based safety standards for laser exposure at the ultra short pulse lengths. Such standards should be determined in light of a basic understanding of the molecular biology of laser-tissue interactions. This work will begin to fill an important void in the current understanding of laser-tissue interaction at the molecular level. The results are directly applicable to the establishment of scientifically relevant safety standards for short pulse, high-energy laser exposures and to the basic science knowledge required to develop rational prophylactic and treatment strategies. Also, an *in vitro* human model for assessing laser-light damage to tissue is desirable for scientific, political and fiduciary reasons. An example of potential clinical applications that might be developed from the basic scientific understanding garnered includes anti-oxidant treatment or other prophylaxis for responses to laser-induced oxidative damage.

Hypothesis

Exposure of human melanocytes grown in tissue culture to high-energy, ultrashort pulse, laser energy will result in genetic induction measurable by differential protein expression as determined by immunologic screening of western blotted of protein samples.

Definition of Terms

Please note: definitions given are relevant to the context of this paper, though they may have wider or sometimes differing meanings in other contexts.

Apoptosis: "programmed cell death"; death precipitated by an orderly sequence of biochemical events encoded for within a cell's DNA. It is an essential element of biological control in an organism which is tightly regulated and triggered by specifically signaled events (Fairbanks 1999).

Bioinformatics: computational manipulation of large biologically relevant data sets to facilitate analysis and enable human comprehension of relationships between elements of the data set (Goodman, 2002).

Chirp: a.k.a. group velocity dispersion (GVD) broadening, an intentional manipulation of the self-focusing properties of a light transmission medium in order to decrease the self-focusing properties of that medium (Rockwell, 2001).

Chromophore: chemical which absorbs light, resulting in absorption of the light's energy (Devlin, 1997).

De novo synthesis: anabolic reactions producing a biomolecule from its substituent atoms and related compounds, as opposed to salvage pathways (Devlin, 1997).

Electrophoresis: a procedure in which voltage is applied to charged molecules, inducing them to migrate (Fairbanks, 1999). It was used in this research to separate proteins on the basis of size. See SDS PAGE, below.

Fluence: amount of laser energy in a beam, in a pulsed beam it is dependent upon the beam's wavelength, intensity, pulse width, and pulse repetition frequency (Rockwell & Hammer, 1999).

Fold number: expression of differential gene expression as a numerical factor, i.e. a tripling of the amount of protein produced by a given gene would be 3x.

Laser induced breakdown (LIB): a nonlinear effect consisting of formation of a plasma in a light transmission medium due to localized absorption of a portion of the incident energy (Rockwell, 2001).

Group velocity dispersion (GVD) broadening: a.k.a. chirp, an intentional manipulation of the self-focusing properties of a light transmission medium in order to decrease the self-focusing properties of that medium (Rockwell, 2001).

Inducible: ability of transcription and translation of a gene to be influenced by a stimulus, resulting in a change in the amount of gene product produced by the cell (Roy, 2001).

Laser (Light Amplification by Stimulated Emission of Radiation): laser light consists of a coherent (parallel) beam of photons of equal energies (Webster's, 1994).

Lithotripsy: a clinical photoacoustical application in which mineral deposits (such as kidney stones) are broken apart using sound waves generated through use of a laser (van Gemert, 1989).

Melanin: a complex tyrosine-derived pigment that acts as a protective primary broad-spectrum chromophore; typically found in skin cells (Prota, 1988).

Melanocytes: dendritic (branched) cells found in the epidermis of skin that produce the light-protective pigment melanin (Montagna, 1956).

Monoclonal antibodies: single antibodies, produced in cloned cells; their extreme specificity for molecular binding makes them useful for assaying for the presence of the molecule the antibody recognizes (Goldsby, 2000).

MW: molecular weight, expressed in terms of kilo Daltons (kDa), each of which is equal to 1,000 times the weight of a carbon atom (McMurry, 1984).

Nd:YAG laser: denotes a laser whose crystalline body consists of a rod of neodymium-doped yttrium/aluminum/garnet crystal (Fukuda, 2002).

NCBI: National Center for Biotechnology Information, a searchable on-line biotechnology data base maintained by the National Library of Medicine under the auspices of the National Institute of health. Accessible at http://www.ncbi.nlm.nih.gov/

Nonlinear effects: a change to the wavelength or fluence of the transmitted light that can occur when light propagates through a non-vacuum medium, resulting in an amplification or concentration of the energy (Rockwell, 2001).

Photoablation: use of a laser beam to vaporize a substance at the point of incidence (van Gemert, 1989).

Photoacoustic: referring to the use of a laser beam to generate sound, as in the generation of shock waves (van Gemert, 1989).

Photobiostimulation: causing a change in a biological process by stimulating that system with a laser beam (van Gemert, 1989).

Photochemical: referring to interaction of a laser beam with a specific chemical (van Gemert, 1989).

Photothermal: referring to the generation of heat by causing a laser beam to interact with a substance (van Gemert, 1989).

Proteomic: referring to the protein content of a cell. Proteomics is the study in parallel of levels, localization, and post-translational modification of proteins produced by an organism (Goodman, 2002).

PVDF: polyvinylidene difluoride, a synthetic material used in the manufacture of membranes for Western Blotting, as an improvement over nitrocellulose membranes (BD Transduction Labs, 2002).

Retinal pigmented epithelia: a layer of highly melanated cells behind the retina of the eye which serve to absorb light, preventing its reflection from the rear of eye (Nicolas, 2000).

SDS PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. SDS is a detergent that dissociates noncovalently bound proteins, and coats them with negative charges, so they will migrate at different rates based roughly on molecular weight, toward the anode in electrophoresis. PAGE is a gel medium in which electrophoretic separation is commonly carried out (Fairbanks, 1999).

Self focusing: a nonlinear effect in which the refractive properties of the transmission medium cause unintended focusing of the transmitted light beam (Rockwell, 2001).

Ultrashort Pulses: laser pulses of less than one nanosecond duration (Thomas, 2001).

Western Blot: procedure that transfers the protein in an electrophoretic gel to a filter while keeping the protein in the same position on the filter as it occupied in the gel; individual proteins are then identified by immunoassay (Weaver, .1999).

Assumptions

Melanocytes are primary mediators of dermal light exposures (Montagna, 1956). It is assumed that skin-derived melanocytes are genetically inducible by light and are therefore an appropriate cell lineage for experimentation.

Delimitations

Protein expression products were measured at a single 24-hour endpoint due to the expense (approximately $5,000 per run) currently associated with parallel immunologic blotting technologies. While it is assumed the 24-hour endpoint is relevant, it provides a single point in time, i.e. a "snap-shot" of the cellular response to laser exposure. Ideally, further investigation would consider a continuum of endpoints such as at 1-, 3-, 6-, 9-, 12-, 24-, and 48-hours, etc. post exposure.

A single experiment and corresponding control sample were subjected to the PowerBlot™ immunologic assay. Ideally three or more identical sets of experiments would have been accomplished in order to demonstrate reproducibility of the experimental results. A contrasting viewpoint however, is that data from this experiment constitutes pooled data set. It is pooled in the sense that if microfluidic techniques and protein amplification techniques were available it would be possible to perform the analysis described below on the protein contents of a single cell. Because this is not possible with current technology, the data described below is the result of an experiment conducted on over 90 million cells subjected to the same conditions in parallel. Specifically, the experimental and sample sets consisted of 11 and 12 plates, respectively, with 96 wells each, thus there were 1056 individual exposure experiments and 1152 controls. In each case the independent variable, laser fluence, maintained constant so that any variations in placement angle, etc. would average out across the 90 million or more cells sampled (see CHAPTER III METHODS, below). Additionally three independent immunoassays were performed to validate the reproducibility of the Western Blots performed.

The experimental cell line, A2058, (ATCC #CRL-11147) was derived from an epithelial skin malignancy (American Type Culture Collection, Manassas, VA). Malignant cells are altered cells that likely have multiple, unknown genetic differences when compared with nonmalignant cells. These differences are not, and indeed cannot, be fully understood due to limitations in the basic scientific knowledge of the numerous mutations and resultant changes that occur in various cancers (Murphy, 1998).

CHAPTER II

REVIEW OF LITERATURE

Review of Previous Research

Introduction

An exhaustive search of the literature revealed no other efforts to understand laser-tissue interactions using a proteomic approach. The only other similar work was done by John Obringer (personal communication) using two-dimensional SDS-PAGE to separate proteins and matrix-assisted laser desorption/ionization mass spectroscopy (MALDI Mass Spec) to identify proteins of interest in laser exposed ocular cells which laid the basis for the plausibility of this project. Therefore, research opens a new realm of investigation into laser-tissue interactions. New aspects include the focus on skin rather than optical tissue, the use of an in vitro model, and the focus of the investigation at the cell-molecular level using proteomics. Due largely to technical limitations, previous research has focused on laser-tissue interactions at the histological, i.e. tissue level. Most often this has been in corneal and retinal tissues, though there has been some work on dermal tissues (Johnson, 2001; Roach, 2001; Stuck, 1981; Lund, 1970; Zuclich, 1995).

A large void currently exists in the current understanding of laser-tissue interactions at the cell and molecular levels. Specifically, previous research has focused on the (macroscopic) histopathological minimum visible lesion (MVL) threshold of various laser exposures in consideration of multiple variables (Cain, 1995, 1999) while failing to consider possible long-term sequelae of sublethal (non-lesion forming) irradiation or as occurs to the cells in the margins of lesions. Biochemical studies have investigated free radical formation in the ocular melanosomes in retinal pigmented

epithelia (pigmented cells behind the retina) which are hypothesized to lead to oxidative damage as one mechanism capable of producing sublethal sequelae of biological significance (Glickman et al., 1996a, 1996b, 1995, 1993, 1992, 1989, Lam et al., 1992).

Laser-tissue research in general is complicated by the number of variables involved, many of which concern technical aspects of laser illumination and the biological model used. Recognizing a further complicating factor relevant to previous laser-eye interaction research, Johnson notes, (2001) "Extrapolation from [eye to skin] is not generally advisable, as the structure and function of each tissue is different. The layers that comprise the skin are different in composition, thickness, and optical properties in comparison to the cornea." Additionally, it is not always true that skin damage requires higher energy fluence than eye tissues, at least in the infrared portion of the electromagnetic spectrum (Rico, 2000). In the 1990s an extensive effort was made to revise laser safety standards. This effort examined histological effects as a function of numerous variables, such as wavelength, pulse duration, total number of pulses, time between pulses etc. so that "the list of important variables expands to create a large matrix of possible experimental conditions" (Thomas, 2001). Due to technological limitations however, no attempt was made to undertake a comprehensive examination of the effects of irradiation at the molecular level and develop a similar matrix.

The research described here attempts to gain a preliminary understanding of some details of laser-tissue interactions at the biomolecular (protein) level of cellular physiology. Nonetheless, detailed research at the histological level is not only relevant but also necessary to a full understanding of laser-tissue interactions. As has been noted, the research described herein was conducted on monolayer tissue cultures. While tissue

cultures may serve as a model, application of the knowledge gained will be largely in the context of living organisms with fully developed tissues and organs. Thus previous research sets not only the background but also the very foundation for understanding how laser light interacts with the skin of living persons.

Mechanisms of Biological Interaction

Five different laser-tissue interaction mechanisms are used clinically in the laser treatment of medical patients: photothermal, photoablative, photoacoustical, photochemical, and photobiostimulation (van Gemert, 1989). The first four of these techniques use the laser to kill and/or remove target tissues. Photothermal mechanisms make use of heat production when laser light is absorbed by chromophores such as blood or cholesterol. The heat produced can be used to cause irreversible tissue damage such as cauterization of pathologic tissue or in control of hemorrhage. Heat can also be used to weld tissues such as blood vessels or nerves together. Photoablation mechanisms make use of a higher level of heat production than photothermal mechanisms such that vaporization of the irradiated tissue occurs. The removal of arterial plaques is an example, as are laser eye surgeries. The depth of ablation must be controlled within microns to prevent unintended damage. Control of ablation is managed through selection of wavelength and is a function of pulse duration as well. Photoacoustical or photomechanical interactions are used to fragment tissues such as corneal cataracts and ureter stones (lithotripsy). In this application, the high electric intensities produced by the laser pulse results in formation of plasma bubbles, with resultant shockwaves that shatter or fragment the target tissues. Photochemical therapies use photosensitizing

chemicals injected into the body. Laser irradiation reacts with the accumulated photosensitizing chemical to irreversibly damage target tissues. For example, injection of hematoporphyrin derivative (a decomposition product of hemoglobin) which accumulates in tumors, can be used to destroy tumor cells by subsequent laser irradiation. The previously mentioned mechanisms of laser-tissue interaction cause direct tissue destruction by thermal or locally-induced mechanical means. The final laser-tissue mechanism in clinical usage is photobiostimulation. This technique relies on laser-tissue interactions to stimulate faster healing by non-thermal irradiation at low power (van Gemert, 1989). Due to the nonlethal aspect of such clinical laser applications, these applications are most closely related to the research described in this thesis. Notably, van Gemert states "little is known about the biological responses of tissues following laser irradiation." That statement is still true today. Such biological responses are the subject of this research.

Photochemistry: Physical Mechanisms Initiating Biological Damage and Response

Laser (Light Amplification by Stimulated Emission of Radiation) light consists of a coherent beam of photons. When these photons interact with the matter of biological systems, the resulting photochemistry may have a physiologically relevant effect on the organism. The nature of the changes induced by photochemical interactions depends on several variables. Of primary importance are the wavelength of the light and the chromophore that absorbs the laser energy. Different wavelengths affect different chromophores, potentially causing damage to those cellular compartments that contain the target chromophore. Ionizing radiation (X-rays, gamma rays) are well known to

cause major nuclear DNA lesions by initiating strand breaks, indicating that DNA is a primary biological chromophore at these wavelengths. For UV (ultraviolet light) in the 220-313 nm region, including germicidal 254 nm lamps, the action spectrum for cytotoxicity follows the DNA absorption spectrum, indicating that DNA is the primary chromophore at these wavelengths as well (Jones, 1985). UVA (193) nm radiation primarily affects the plasma membrane, possibly by 193 nm absorbance by amide bonds and aromatic groups in the membrane-bound proteins (Kochevar, 1990). With longer wavelength light such as 1540 nm, damage appears to be photothermally mediated by melanin absorption as well as water absorption (Roach, 2001).

Penetration of Laser Light in Tissue

Another important variable in laser-tissue interactions is the depth the laser light is able to penetrate. Skin is both highly organized and highly variable in the thickness of its component layers in different parts of the body (Montagna, 1956). The depth laser light is able to penetrate depends on several variables. The first is the wavelength of the incident light. van Gemert (1989) reports that tissue penetration varies between less than one cell thickness at wavelengths of 200 nm (ultraviolet) and 2940 nm (infrared) to a maximum of approximately 1 cm around wavelengths of 700 nm. Light absorption by tissues is mediated by different mechanisms, which are also dependent on wavelength. Water and protein absorption is the primary mode for UV light, while chromophore absorption is more predominant in the visible and near IR, and absorption by water predominates again in the further IR, i.e. greater than or equal to 1300 nm (Query, 1991).

It is widely accepted that the degree of melanization (darkness) of skin affects the penetration of light, though the quantification of this effect remains poorly characterized.

Biological Implications of Nonlinear Laser Light Propagation

"Nonlinear effects" are a possible mechanism in mediating laser-tissue interactions. Nonlinear effects occur when light propagates through a non-vacuum medium, resulting in a change to the wavelength or fluence of the transmitted light, i.e. an amplification or concentration of the energy. For biological systems this means a reduced amount of incident energy is required to cause damage when a nonlinear propagation effect occurs in the biological medium, such as the vitreous humor of the eye (Cain and Jost, 1999). Nonlinear effects include laser-induced breakdown (LIB), self-focusing, group velocity dispersion (GVD) broadening, a.k.a. chirp (Rockwell, 2001) and multiple photon or multiple quanta absorption producing frequency upconversion. For example, absorption of two 1064 nm photons can result in the release of an "upconverted" 532 nm photon, two of which could then theoretically be upconverted to one 266 nm photon. In research on optical tissues nonlinear effects have been shown to have considerable biological significance (Rockwell & Toth, 1999).

The author is unaware of any research on nonlinear propagation effects of laser irradiation in skin. Self-focusing and GVD are unlikely to occur in skin due to the dispersive and absorptive natures of skin compared to eye tissues, which by design readily transmit light. The tendency during light transmission through skin is for scattering and absorption which necessarily limit the possibility for self-focusing or GVD.

Laser-induced breakdown, in which laser energy is absorbed by the transmission medium itself and results in the formation of a plasma remains a possibility in skin (Cain, 1996). If LIB occurs in skin it is a potential mechanism for inducing biological effects, including DNA damage. Also, multiple photon absorption and frequency upconversion of the incident photons could have significant, possibly mutagenic effects as well (Leavitt, 1997). Upconversion of 532 nm light would result in the next higher harmonic, 266 nm, which is in the UV range. Formation of pyrimidine dimers is one widely documented mechanism of DNA damage resulting from exposure to UV light. Cao, et al. (1993), posit this as the physical phenomenon for possible mutagenic effects of 532 nm light, saying "Exposure of thymine and DNA to high-intensity 532 nm pulsed radiation from a Nd:YAG laser resulted in cyclobutylpyrimidine dimers, which were measured by the method of high performance liquid chromatography." With DNA involvement as a chromophore over a wide portion of the light spectrum due to both the primary wavelength of incident light and nonlinear effects, it is likely that DNA damage repair mechanisms would be upregulated at some point following laser-tissue interactions.

Western Blotting as a Means of Biological Inquiry

Proteins carry out the majority of chemical activity necessary to life cellular life, serving as structural members of cells, enzymes, and regulating life processes. Western blotting and subsequent immunologic identification of proteins is one method of assaying the proteomic (comprehensive protein) response of a biological system to an environmental stimulus (Kiechle, 2002).

The central dogma of modern biology is that all the chemical/biological information required by a life form is stored in DNA, which is transcribed to RNA and then translated to protein. According to this paradigm the potential response of a biological system to any variation in its environment is encoded in the DNA. The response to an environmental stimulus, such as exposure to a laser beam, may require changes in the transcriptional and translational levels to mediate the required response. Monitoring changes in the transcription levels of various messenger RNAs and the corresponding translation of the proteins they code for would therefore give direct indications of the cell's specific response to the environmental stimulus at the molecular level. Monitoring of protein expression patterns can be accomplished by western blotting techniques in conjunction with immunologic identification of the proteins and bioinformatics analysis of the resultant data set.

Ideally there would be a quantitative means of measuring the transcription, translation and residency times for RNA transcripts and the proteins they code for. In lieu of such a robust system, multiple parallel western blots of cellular extracts are used in this research to query the biological response to laser exposure at the level of translation. By examining the differential protein expression patterns it is hoped that insight in to the physiological state of the cells can be ascertained, including but not limited to the amount and type of damage/perturbation the cell has undergone. If this is possible, it may further be possible to predict whether the damage has induced an increased likelihood of future biological perturbation such as mitotic misregulation (an alteration of the control of cellular replication) due to mutagenesis (causing a mutation in

the DNA) or a change that will result in the cell undergoing programmed cell death (apoptosis) outside of its normal developmental program.

In this research the BD PowerBlot™ system, BD Transduction Laboratories, Lexington, Kentucky, was employed to provide proteomic analysis of laser- irradiated melanocyte extracts. The BD PowerBlot system assays proteins against 859 monoclonal mouse antibodies individually specific for physiologically relevant proteins known to be mediators of processes such as apoptosis, cell adhesion, transcription and translation regulation, cell cycling control and machinery, membrane interaction, organelle function, cytoskeleton components and regulation, nuclear transport, etc. Protein isolated from control versus experimental cells is densiometrically analyzed after contact with the array and compared for significant differences. These differences are presumably indicative of the metabolic differences between lased and unlased cellular responses, and equate to the cell's response at the protein level to laser -induced alteration in cellular physiologic state. Relative changes in expression are verified by comparison of control (sham-exposed) and experimental lysate samples on the same gel.

Summary of the State of the Art

The current state of the art in understanding laser-tissue interactions has resulted in the establishment of safety standards for ultrashort laser pulses, largely based on minimum visible lesion (MVL) experimentation in the rhesus monkey ocular model. Maximum permissible exposures (MPE) are thus based on histological data, i.e. data that looks biological damage at the tissue level. There is currently little data available at the molecular level as to the bioeffects of laser-tissue interaction, though histological evaluation of porcine skin is in progress (Roach, 2001).

CHAPTER III

METHODS

Procedures

Cell Lines

A human melanoma line "A2058" (ATCC #CRL-11147), obtained from the American Type Culture Collection, Manassas Virginia, was used in this investigation. The experimental line was chosen on the basis of presumed relevance of the cell type (epithelial tissue derived), to genetic induction in response to exposure to light. A2058 is an amelanotic melanoma line (the cells do not constitutively produce melanin). The line was established from a lymph node metastasis removed from a 43-year-old Caucasian male with malignant melanoma. The cells are characterized as highly invasive (ATCC #CRL-11147 Catalog Description) and proved to multiply with extreme rapidity in tissue culture. Doubling time was approximately 16 hours (Dr. Ann Cox, personal communication).

Media

Culture medium for A2058 cells consisted of 90% Dulbecco's modified Eagle's medium with L-glutamine and sodium pyruvate, adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose with 10% volume per volume fetal bovine serum (ATCC #CRL-11147 product information sheet). Sterility of media was ensured by passage through a disposable .22μm vacuum filtration unit.

Culture

Frozen cell lines obtained from ATCC were thawed upon receipt in a 37°C water bath, and then propagated in monolayer tissue culture flasks in the appropriate medium. Cultures were maintained at 37°C in a humid 5% CO_2 atmosphere incubator. Adherent cultures were allowed to grow to confluency in a 25 cm^2 flask, then enzymatically dissociated with .025% trypsin, subcultured in 75 cm^2 flasks and allowed to grow to confluency. The cultures were subcultured again and plated in Falcon® MicroTest™ 96 Well flat bottom gas-plasma treated sterile tissue culture trays, (6mm diameter per well, microtiter plate format) using a 1:4 to 1:6 split ratio. Plated cells were allowed to grow to confluency (growth so that cells touched each other) in preparation for laser exposure. Average cell density at the time of laser treatment was 3750 cells/mm^2. Given the 6 mm diameter of the wells in the tissue culture trays, this works out to approximately 106,000 cells per well. Eleven 96-well trays (1056 individually treated wells of cells) were exposed to the experimental laser illumination and twelve trays were used as shams (1152 control wells of cells).

Laser Irradiation

Prior to laser irradiation 96-well cultured trays were decanted and refreshed with 50 μL fresh media to standardize pH and fluid levels in the wells. Batches of prepared 96 well trays were maintained at 37°C in an incubator while awaiting laser illumination.

An EKSPLA SL 312 T pico-second neodymium-yttrium-alluminum-garnet (Nd:YAG) laser was used to illuminate the cells. The beam was aimed and focused with beam steering and shaping optics so that the beam shape and size precisely matched the

wells in the tissue culture trays. Fluence levels were determined using a Molectron Opti-Mum 4001 joulemeter ratiometer with Molectron J50 heads with Molectron model JA-YAG-50 diffusers. Computerized control of a motorized Velmix Unislide 2-axis translation stage and NM Laser Products shutter system permitted precise positioning of the wells relative to the laser beam for raster scanning of trays and control of laser fluence through the cultures. Cells were treated directly in the 96-well trays they were cultured in.

Irradiation was with light of 532 nm wavelength (light green). Irradiation characteristics of the 532 nm pulses were 120 picosecond pulse length, measured full-width half maximum (FWHM), at 10 cycles per second (Hz). Target energy fluences (total energy) were achieved by controlling the duration of shutter opening so that the desired number of pulses, (at 10 per second) was permitted to impinge upon the cells in each well. Back reflection was limited by use of an absorbent black surface on the translation stage which the cell culture trays rested on during laser irradiation.

Total target energy was initially desired to be an ED_{50} dosage, i.e. one which would result in death of 50% of the cells at a 24-hour endpoint. Using an MTT (tetrazolium salt) viability assay (Berridge, 1996), it quickly became apparent that the polystyrene culture trays would blacken and burn causing outright ablation of the monolayer culture before an ED_{50} dosage could be achieved at these wavelengths and pulse widths. Comparison of sample to control in these dosimetry experiments consistently showed 93-98% viability of laser-exposed cells in non-burned trays compared to controls exposed at 532 nm in the range of 55-20 mJ, increasing pulses up to the burn threshold (data not shown). Subsequent experimentation was subject to

considerable laser instability at higher operating (peak pulse) powers, resulting in a reduction of peak power to 45 mJ and longer shutter opening times to achieve a desired constant total energy. At these peak powers, the threshold for burning of the polystyrene for 532 nm light was 25 pulses. This value was determined by 100x microscopic examination of plates looking for burn marks. Based on this information, 20 pulses was the value selected to maintain a constant total energy and remain below the polystyrene burn threshold. 20 Pulses with peak energies of 45 (+/- 3) mJ per pulse were used yielding a total fluence per well of 1.9 Joules in the experimental samples. Table 1, below, shows the amount of energy absorbed by dry tissue culture trays, and trays with water, media only, and media with cells. The experimental samples were laser treated while bathed in 50 μL of fresh media. Accounting for the absorbance of the media and tray, we see that the experimental cells absorbed 16% of the energy in the laser beam. At a total fluence of 1.9 Joules per well (the experimental exposure), the cells in each 6 mm diameter well absorbed .304 J of energy. At a density of 3750 cells per mm^2, each of the approximately 106,000 cells in the well would have absorbed 2.87 x 10^{-6} Joules.

10 pulses/well, @ 10 Hz, (No burns in empty tray)	Dry Plate	Distilled Water, 50μL	Media, 50μL	50μL Media w/ Cells
Fluence, mJ/pulse, n=100	47.0 +/- 1.1	47.1 +/- 2.0	45.0 +/- 2.5	45.0 +/- 2.5
Transmittance, mJ/pulse, n= 40-80	45.0 +/- 0.5	46.0 +/- 1.5	36.7 +/- 1.5	30.6 +/- 1.7
Absorbance, mJ/pulse (=Transmittance – Fluence) *Range	2.0 *3.6-0.9	1.1 *3.6-0	7.3 *12.3-4.3	14.4 *18.6-10.2
% Absorbance (Absorbance / Transmittance x 100)	4.3 %	2.3%	16.2%	32.0%

Table 1. Plate Dosimetry: Laser Absorbance, 532 nm, 3.0 ns [sic] pulse

Control Procedures

Cells grown as controls were exposed to the same conditions including transfer to the laser room, exposure to open air for the shoot period, and movement on the x-y translation state in order to expose both samples and controls to the same open air conditions, noise, and electromagnetic fields, etc. The controls, however, were not exposed to laser light, as the laser apparatus was turned off for the period of time the controls were sham exposed to the open air x-y translation stage conditions.

Harvest and Cell Lysing for BD PowerBlot™ Assay

Twenty four hours post-treatment, the harvest protocol was initiated. The harvest protocol consisted of washing twice with 50 µL Hanks' balanced saline solution (HBSS), once with 50 phosphate-buffered saline (PBS), followed by freezing decanted cells overnight (-64°C) in the covered and sealed 96-well trays they had been grown and irradiated in. The freezing was found helpful in that it initiated cellular lysis and release from the trays, exposing more surface area to the lysis buffer. Lysis buffer consisting of 10mM Tris (pH 7.4), 1 mM sodium ortho-vanadate, and 1% SDS was obtained from BD Transduction labs and used for the following cell lysis procedure:

1) Lysis buffer was heated to just below boiling in a double boiler.
2) The 96-well tray to be harvested was placed directly on a wet block heated to boiling.
3) 20 µL lysis buffer per well was added by 8-channel pipet to the first column of cells.
4) The cells were lysed by repeated pipetting and the lysate/buffer mixture was transferred to the next column of cells. This process was repeated for four columns, and

then the cell lysate was pipetted into a sterile disposable plastic collecting trough. No more than four columns could be done without adding additional buffer due to evaporative losses and the formation of bubbles in the micropipette tips. This process was repeated until all cells were harvested.

5) Collected lysate was transferred to a 50 mL polystyrene centrifuge tube.

6) The lysate was heated in a boiling water bath for 30 seconds.

7) To shear cellular DNA the warm lysate was homogenized 15-30 seconds with a Tissue Tearor™ homogenizer (Biospec Products, Inc.)

8) Homogenized samples were frozen at -65°C awaiting shipment to BD Transduction Labs for BD PowerBlot™ analysis.

9) Samples stored at -65°C were packed on dry ice for overnight express shipment to BD Transduction Labs.

After and between uses the Tissue Tearor™ was cleaned by sequential 30 second runs in the following series of rinse solutions:

1) 70% Isopropanol,
2) 70% EtOH
3) Distilled H_2O
4) .1 M NaOH
5) Distilled H_2O
6) NaCl, saturated solution
7) Distilled H_2O
8) 70% EtOH
9) Distilled H_2O

BD PowerBlot™ Materials and Methods

The following procedures were performed by BD Transduction Laboratories, Lexington, Kentucky. Descriptions of procedures conveyed below were provided by Ms. Bryden Heywood at BD Transduction Labs.

Samples and controls were received, thawed and quantitated using the Pierce BCA reagent colorimetric assay to ensure adequate sample quantity. Protein concentration in solution was equalized by dilution and the samples are loaded for SDS PAGE. The separated proteins on the PAGE gels were transferred to PVDF membranes (western blotted) so that samples of the proteins were transferred to the surface of the membrane, replicating their exact relative positions on the PVDF membrane. This allows correlation of lane number and molecular weights (as determined by electrophoresis) with protein identification obtained from the next steps. The membranes containing the electrophoretically separated proteins are then probed with specific mouse monoclonal antibodies. The complete list of these antibodies can be found in Appendix A. Antibodies bind to those proteins in the blots for which they are specific, while antibodies not bound are rinsed away. The antibody-protein complexes are then incubated with fluorescently tagged anti-mouse antibodies. Binding of the fluorescently tagged antibodies allows the bound proteins to be visualized under the proper wavelength of light. The data is captured as an electronic photograph when membranes are exposed to infrared light and read with the Odyssey™ Infrared Imaging System. PDQuest software (by Bio-Rad) automates the spot-finding and spot-matching functions that correlate SDS Page information (molecular weight) with Western blot data (immunologic binding, spot size). Finally, bioinformatics techniques are used to generate confidence levels based on

reproducibility, fold change and spot intensity, and present the data set in an MS Excel spreadsheet (see Appendix B).

The Western blotting protocol is listed below, per the BD Transduction Labs PowerBlot™ Service product information sheet, (Bryden Heywood, January 2002). Prior to this procedure protein concentrations were quantitated using the Pierce BCA reagent colorimetric assay. Protein concentrations were then equalized by dilution and rechecked.

"Western blotting - all steps are carried out at room temperature.

a) Samples are run on Criterion gels purchased from BioRad, and are 5-15 % gradient SDS-polyacrylamide, 1mm thick. A gradient system is used so a wide size range of proteins can be detected on one gel.

b) 200 µg of protein is loaded in one big well across the entire width of the gel. This translates into ~15 µg per lane on a standard 25 well gel. The gel is run for approximately an hour at constant milliamps.

c) The gel is transferred to Immobilon-P nylon membrane (Millipore) for two hours at 200 milliamps. We use a wet electrophoretic transfer apparatus TE Series from Hoefer. One hour in 1:1 LI-Cor blocking buffer: TBS.

d) Next, the membrane is clamped with a western blotting manifold that isolates 40 channels across the membrane. In each channel, a complex antibody cocktail is added and allowed to hybridize for 45 minutes.

e) The blot is removed from the manifold, washed and hybridized for 1 hour with Alexa Fluor 80 goat anti-mouse IgG. All our antibodies are mouse monoclonal so only one secondary is needed.

f) The membrane is washed [and] dried. Images are captured on LI-COR [sic] infrared scanner.

g) MW Standards – Standards are composed of an antibody cocktail added to lane 40 of the PowerBlot gels

P190 Glued	190
Adaptin beta	160
STAT-3	92
Mek-2	46
RACK-1	36
GRB-2	24
Rap2	21 "

Statistical Procedures

The following information was provided by BD Transduction Laboratories, Lexington, Kentucky, regarding statistical analysis of samples and interpretation of resulting data labels: Fold change is a "semiquantitiave value" representing the general trend of protein changes for the experimental sample relative to the control. Protein expression levels were determined by the following procedure: Separated proteins from SDS-PAGE are transferred to PVDF membranes by Western Blot, assayed with cocktails of mouse-derived antibody cocktails, and rinsed. The mouse antibodies are then incubated with fluorescently tagged anti-mouse antibodies. Binding of the fluorescently tagged antibodies allows the bound proteins to be visualized under the proper wavelength of light. The data is captured as an electronic photograph when the membranes are exposed to infrared light and read with the Odyssey™ Infrared Imaging System. PDQuest software (by Bio-Rad) automates the spot-finding and spot-matching functions that correlate SDS Page information (molecular weight) with Western blot data (immunologic binding, spot size). Raw data was the total intensity value of a spot.

Calculation of Fold Change

The raw values determined as described above were normalized by dividing the raw quantity of a spot by the total intensity value of all pixels in an image multiplied by 1,000,000. The ratio of normalized quantity for experimental samples to the corresponding spot on the control sample is the protein expression fold change. Specifically, differential protein expression, i.e. "fold change" was calculated by dividing the signal strength of the experimental sample by that of the control sample for each run

of the Western blots. "+" Indicates an increase in expression of a protein in the laser exposed samples., while "-"indicates a decrease in expression, and div/0 indicates that the protein was not detected in the experimental but was detected in the controls or it was detected in the control sample but not the experimental sample, so that calculation of fold change would result in irrational number (division by zero). Thus + div/0 means the protein was detected in the laser exposed sample but not the control, while – div/0 indicates the protein was detected in the control but not in the laser-exposed (experimental) sample.

Protein fold expression confidence level was calculated for each protein based on a combination of signal strength and the absolute value of the fold increase itself, as well as whether all three Western Blot runs agreed in the direction of fold change (i.e. did they all increase/decrease, or was one run in conflict with the others). Confidence level five was assigned to changes greater than 2 fold in triplicate from good quality signals. This is the highest confidence level. Changes from good quality signals in triplicate with fold changes of 1.5 to 1.9 were assigned confidence level four. Changes of 1.25 to 1.5 in triplicate were assigned confidence level three.

Fold changes assigned confidence levels five through three were all in agreement to direction of change and from strong or good signals. In contrast, changes in triplicate from low signals were listed as level two. Changes greater than 2-fold but observable only in duplicate, were assigned confidence level one. Though from good quality signals, one of these runs either showed less than .25-fold change or was in conflict as to the sign of the fold change. Though of lower confidence, the protein signals labeled confidence level two and one are still valuable as they show the direction of expression change. Fold

changes represented as div/0 in the appendices represent the presence verses absence of a protein, in which case the fold change cannot be calculated mathematically because division by zero is not possible. In essence, these changes represent a profound change in signal strength. Signals with numeric levels below 30,000 as read by the Odyssey™ Infrared Imaging System, were deemed low signals. "Comparisons based on low signals can be inconsistent" (BD PowerBlot™ Service Technical Data Sheet). Proteins with a predominance of low signals in both the experimental sample and control sample were assigned confidence level two, and are considered less reliable due to the greater potential for error in calculation of fold expression at the lower confidence levels.

CHAPTER IV

RESULTS

Summary of Results

Twenty-four hours post exposure, 532 nm laser-exposed human melanocyte cell protein lysates were examined by an immunologic assay and compared to sham exposed controls to assess changes in protein expression patterns. Tabulated data from the BD PowerBlot™ immunologic assay of protein expression profiles is presented in Appendix B. Of 859 proteins assayed (Appendix A), 95 were identified in the experimental and control samples (Appendix B). Of these 95 proteins, 43 showed a trend of increased abundance (expression) while 42 were less abundant. Confidence levels of five through one were assigned using BD Transduction Lab's bioinformatics protocols, with five being the highest confidence. See statistical procedures, chapter III, for explanation of confidence level assignment. It was noted that 15 of the 43 proteins with increased expression were of the lowest confidence level. With this in mind it can be seen that only 17 of 52 signals with confidence levels five through three showed positive fold changes in the experimental sample. There was a trend toward decreased expression in proteins with good or strong signals whose fold change correlated between the three runs of the Western Blot.

Appendix C, protein function table, shows the 95 proteins grouped by similarity of their primary function(s) in the cell. Literature references are provided for each protein in this table.

CHAPTER V

SUMMARY AND DISCUSSION

Summary of Methods

Using a 24-hour endpoint, 532 nm laser-exposed human melanocyte cell lysates were examined by an immunologic assay to assess changes in protein expression patterns when compared to similarly treated controls. Tabulated data from the BD PowerBlot™ immunologic assay of protein expression profiles is presented in Appendix B.

Summary of Findings

Of 859 proteins assayed for (Appendix A), 95 were identified in both the experimental and control samples, or in one of the sample sets. Of these, those high confidence-level proteins whose expression was upregulated are considered as potential candidates for laser-tissue interaction biomarker status. These proteins include Ubc9 (confidence level 5, fold change 7.45), UbcH7 (confidence level 5, fold change = division by zero), and eIF-5a (confidence level 5, fold change 7.57).

Additionally the general physiological status of the cells as can be interpreted from the differential protein expression levels between samples and controls is explained below (see Discussion). By categories of function, protein expression fold changes indicate the cells were poised to increase the level of proteolytic destruction, down regulate mitotic activity, apoptotic signalling pathways, transcription, translation, and vesicular trafficking. Only two DNA repair proteins were discovered by assay and they show decreased expression. Transcription and translation related proteins also show a downward trend of expression, as do cell adhesion and motility proteins. The general

physiologic state at the 24-hour post-exposure endpoint sampled then, is indicative of having dramatically decreased the level of cellular activity, particularly those which would result in advance the cell cycle toward mitosis. Though not specifically measured in this work, the author's experience (see discussion of MTT assays, above) is that the laser-treated cells tend to recover and survive quite well in culture. Therefore, it is hypothesized that this is a temporal set back for the treated cells. Evidence supporting this view is that the mitotic and apoptotic regulation and of the cell appears to have been down regulated along with the other metabolic functions. The cells were not killed outright nor do they appear poised to execute their apoptotic programs at this datapoint. The cells appear to have paused the nonstop mitotic cycle they exhibit as a cancer line and devoted energy to taking care of the damage caused by the laser treatment.

In summary, it was found that the cell was poised to increase protein destruction levels and generally decrease all other metabolic functions, to include mitosis, apoptosis, transcription, translation, and vesicular trafficking.

Discussion

Interpretation of the physiologic state of cells based on limited proteomic data is problematic, at best. Results from this cell line may differ from what would be seen in other cell lines based on their differentiation, as well as differences that occur due to individual genetic variation, health status, etc. Cellular functioning at the protein level is complex due to numerous factors concerning the regulation of protein function within the cell. While it is clear that a protein cannot carry out its various functions within the cell if it has not been expressed (transcribed from DNA to RNA, the RNA then translated to protein), the presence of a protein does not imply that the protein is functionally active in

the cell. Protein functionality is conferred by many factors, including by not limited to proper folding, post-translational modification, compartmentalization, pH, association with required cofactors, dissociation from inhibitors, energy state of the cell, phosphorylation state of the protein and its substrate, upstream and down steam elements in the signalling pathway and of course presence and availability of the appropriate substrate itself (Fersht, 1999; Devlin, 1997). Nonetheless, a number of observed trends were apparent from the data set which provide the observer valuable insight into the possible metabolic state of the cell after laser exposure.

Protein Destruction

Mammalian cell cycle regulation is accomplished mainly by phosphorylation/ dephosphorylation and synthesis/degradation of numerous proteins. Ubiquitin functions in cell cycle regulation. Ubiquitin targets proteins for rapid degradation by enzymatically attaching a lysine residue in the protein to be destroyed. This 'ubiquitination' is a "hallmark" in proteosomes (cytoplasmic complexes of proteases) (Nuber, 1996). Ubc proteins such as Ubc9 and UbcH7 function to catalyze the covalent attachment of ubiquitin to other proteins, mediating their selective degradation (NCBI P51966). Because of their high fold increase and unique role in protein degradation, Ubc proteins present a potential target as a laser-tissue interaction marker deserving further research in this role.

The expression of ubiquitin-conjugating enzymes (Ubc) was noticeably higher in the laser exposed sample than in controls. Ubc9 fold increase was 7.45, and UbcH7 was clearly visible in the experimental sample but was not detectable in the controls, thus fold

computation resulted in division by zero. Both proteins were detected at confidence level 5. Additionally, UbcH6 showed greater than two-fold increased expression, though it was not detected in one of the gels, so was classified as confidence level 1. Thus there appears to be an increase in the Ubc-related expression at the 24-hour endpoint, indicating the cells were poised to increase the level of protein degradation.

Similarly, prenylcysteine lyase is another degradation protein whose expression was increased (confidence level 2, + 1.79 fold change). Up to 2% of proteins in a cell (on average) are prenylated, and their degradation is mediated in party by prenylcysteine lyase (NCBI NP_057381), (Tschantz, 1999). CUL-3, a protein which targets the cell cycle regulating protein cyclin E for destruction was upregulated. Cyclin E advances the cell cycle from G_0 toward G_1 and synthesis stages (Polanowska, 2001). p47A (confidence level 2, + 1.47 fold change) complexes with other proteins to form a multimeric adaptor protein complex (AP-3) that functions in the sorting of integral membrane proteins as they are trafficked between the TGN and destination membranes (Dell'Angelica, 1997). Importantly, the AP-3 complex with which p47A associates with has been implicated by genetic studies to play a role in the sorting of proteins to lysosomes where controlled digestion of macromolecules occurs. Thus the upregulated p47A is involved not only in vesicular transport, but also in protein degradation.

In contrast to the examples above, SNX1, which binds epidermal growth factor receptor and transports it for destruction (NCBI NP_003090), (Haft, 1998), as well as GSTI-π, a glutathione conjugating enzyme important in the clearance of electrophilic (e.g. free radical) cellular toxins (Kano, 1987), (Ali-Osman, 1997), (Zhou, 1997), (Sheehan, 2001), both showed decreased expression. As noted earlier, free radical

(electrophilic toxin) formation is hypothesized as one mechanism capable of producing sublethal laser-tissue interaction sequelae of biological significance (Glickman et al., 1996a, 1996b, 1995, 1993, 1992, 1989, Lam et al., 1992). The differential protein expression trend shows the cell poised to degrade proteins, likely in response to damage resulting from the laser treatment. The author hypothesizes that this stage in the laser treated cells' response is similar to surgical debridement, i.e. removal of dead and foreign matter from a wound (Webster's, 1994). The laser treated cells may well have upregulated expression of ubiquitin-conjugating enzymes (Ubc9, UbcH7, and UbcH6), a glutathione-conjugating enzyme (GSTI-π), and prenylcysteine lyase in an effort to effect, by analogy, "molecular debridement," i.e. the removal of damaged ("dead") proteins from the cell.

Mitotic Regulation

Twenty three of 29 detected proteins involved in the regulation of mitosis showed changes in their expression levels that indicate a decrease in mitotic activity. The ubiquitin-conjugating enzymes (Ubc) as discussed above, all showed trends toward increased expression, which according to Nuber (1996) is indicative of one major mechanism of mitotic regulation, the destruction of key proteins. It is instructive to remember that the subject cells were of a malignant line and so were rapidly dividing (the normal malignant condition) at the time of laser treatment; therefore mitotic regulation was favoring growth and division. For this reason the increased protein destruction which may be inferred from increased Ubc expression levels (above, and Appendix B, C) would likely bring about a decrease in mitosis through destruction of proteins essential to

the continuous up regulation of cell cycle progression seen in cancer. This observation correlates with the majority of other mitotic regulating proteins reported. See Figure 1.

Sam68 (**S**rc-**a**ssociated in **m**itosis 68kDa, confidence level 5, -6.5 fold change) showed a marked decrease in abundance. Sam68 is an adaptor protein that promotes mitosis (Richard, 1995). Down-regulated expression of Sam68 arrests growth of some cells (Lee, 1999). Three different Shc Proteins, which function as early intermediates in the growth factor receptor signalling cascade (Pelicci, 1992), (Chow, 1998), (O'Bryan, 1996), were down regulated as well. Additionally, Csk, a negative regulator of the Src family kinases which the Shc proteins belong to, showed an increased abundance indicating that the Shc proteins in the cell may have been turned off as well as being generally less abundant (NCBI P41240), (Brauninger, 1993). See Figure 1.

RanBP1-27 (Ran binding protein #1, confidence level 5, -3.79 fold expression change) and RanBP1-32 (confidence level 3, -11.48 fold change), are examples of regulators of mitosis whose expression level are decreased. RanBP1 isoforms are associated with signaling processes such as entry/exit from mitosis, and transport of protein and RNA through nuclear pores by means of its association with Ran. Ran is a conserved GTPase which is thought to function in pathways that control cell cycle progression by regulating the transport of protein and nucleic acids across the nuclear membrane (NCBI, P43487), (Hayashi, 1995), (Ren, 1993). RanBP1 binding to Ran stabilizes the GTP-bound "turned on" state of Ran. That Ran (confidence level 1, +6.03 fold change) itself showed a large fold increase while one of its regulators showed an equally large fold decrease may be indicative of a high degree of disruption to normal signaling and control processes that resulted from the laser treatment, or the increased

expression or Ran may be an early sign that the cell is beginning to produce proteins necessary to resume normal mitotic functioning. Due to the magnitude of the other expression changes, I favor the former argument over the latter. See Figure 1.

Another protein group showing a similar change in expression pattern to the Ran/RanBP1 pair is Cdk1, Cdk2 and KAP (fold changes: -1.87, -1.99, +2.81, respectively). Cdk1 and Cdk2 (cyclin dependent kinases) "define a final common pathway for growth factor-induced and antiproliferative signals during the cell cycle" by promoting the G1-S phase transition (Sherr, 1993). KAP (Cdk associated phosphatase) is thought to regulate the phosphorylation state of cdk2 by dephosphorylating it (Hannon, 1994), (Donato, 2002). Thus decreased abundance of the protein (Cdk) that may signal the advance to DNA synthesis (S) phase and an increased abundance of a protein suspected of dephosphorylating it (thereby deactivating the remaining Cdk) points to a decrease in mitotic activity. See Figure 1.

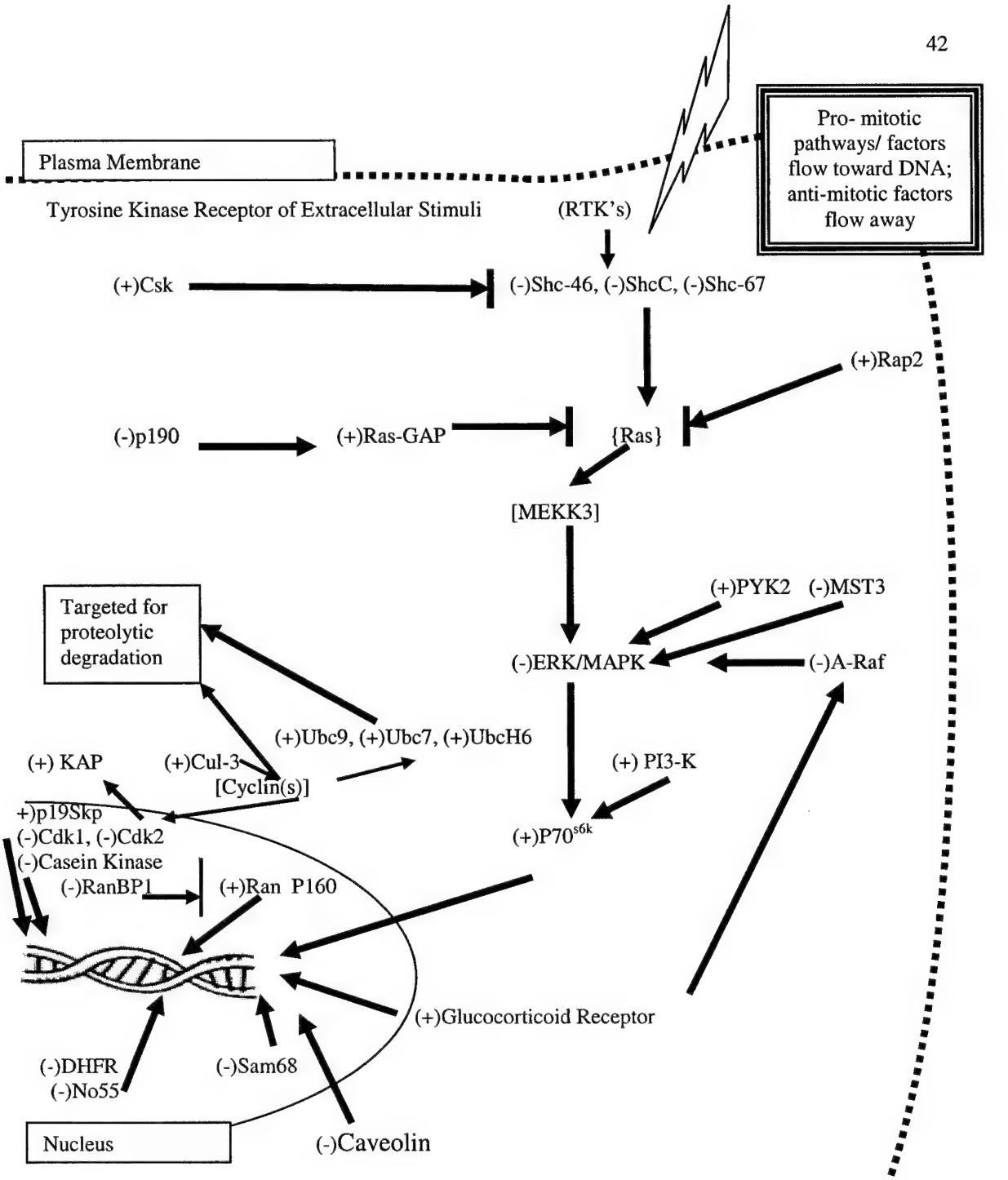
Pan ERK is a positive effector intermediate in one of the pro-mitotic pathways. See Figure 1, below. Additional evidence of decreased cell cycle progression comes from decreased abundance of pan ERK-39 (confidence level 4, -1.79 fold change), a mitogen-activated protein kinase (Sivaraman, 1997), (Cobb, 1995). Various mitogenic signaling molecules, as well as ultraviolet-B light activate ERK (Tada, 2002). Casein kinase Iε, a positive regulator of DNA replication which would be important in S-phase (NCBI P49674), (Fish, 1995), showed a -2.82 fold change (confidence level 3). Reduced expression of this protein further bolsters the supposition that pro-mitotic effectors are show decreased expression.

DHFR, dehydrofolate reductase, performs the critical function of regenerating tetrahydrofolate, a coenzyme for thymidilate synthetase. Formation of purines is tightly regulated at all times and is especially critical during mitosis when the cell is actively and rapidly synthesizing nucleic acid and proteins in order to replicate the genome (Devlin, 1997). This enzyme is essential for *de novo* purine synthesis, and DNA precursor synthesis such as converting dUMP to DTP, making it crucial for assembly of the building blocks the cell needs during mitosis. DHFR is necessary for the formation of DTP, a required substrate for DNA synthesis, i.e. replication. The expression of DHFR (confidence level 3) was down regulated 2.21 fold is strong evidence that the cells were unable to proceed with mitosis at the endpoint examined in this research.

Ras-Gap was upregulated (3.74 fold change, confidence level two). Ras-GAP (ras GTPase activating protein) is a negative regulator (NCBI P20936), (Scheffzek, 1996), of the p21 ras oncogene pathway (Yamamoto, 1984). See Figure 1. A trend toward increased abundance of this negative regulator of the p21 proliferative mechanism provides further support for the observation that mitosis is slowed.

There were protein expression level changes that would tend to indicate a trend toward a pro-mitotic physiologic state. Expression of p70s6k (confidence level 2, +2.07 fold change), a mitogenically stimulated kinase (NCBI P23443), (Grove, 1991), (Romanelli, 1999), and PYK2 (confidence level 2, +2.22 fold change), which functions in the MAP (mitogen activated protein) signalling pathway (NCBI Q14289), (Lev, 1995), are two examples. Both proteins are positive effectors in pro-mitotic signalling pathways. However, the pro-mitotic expression changes (Ran, p70s6k, PYK2, p19Skp1, glucocorticoid receptor, p190) were of lower confidence levels, i.e. levels one and two,

and were greatly out numbered by the differential expression changes which indicated anti-mitotic cellular signalling. See Figure 1 for a diagrammatic integration of the aforementioned mitotic regulation.



Implication: Mitosis, slows/halts, including: nucleotide synthesis, DNA replication, cell division

Apoptotic Factors

Apoptosis-related (programmed cell death) signaling molecules were well represented in the list of proteins assayed for and detected. The trend was for expression changes that reduced the likelihood that an apoptotic pathway would be triggered. Of 12 apoptosis-associated proteins found, nine showed expression changes that would reduce the likelihood of apoptosis, while three showed changes that would increase the likelihood.

Anti apoptotic proteins are described here. LITAF (lipopolysaccharide-induced TNFα factor) is thought to link p53 cell death pathways with TNFα production (NCBI NP_004853), (Myokai, 1999). Its abundance was reduced 2.12 times over that of the control. This change is indicative of a reduced chance of apoptosis by TNFα-induced pathways. hILP (human inhibitor of apoptosis-like protein) functions by inhibiting caspase-3, caspase-7 and caspase-9 (Fig. 2), enzymes which function in the cascade of events that causes the apoptotic effect (NCBI P98170), (Duckett, 1996). hILP (confidence level three) showed a 6.63 fold increase. The average hILP fold increase is suspicious due to the third run having a fold increase of 17 compared to slightly less than 1.5 for the other two runs, but the positive expression trend is clear. The expected result of this expression change is a strong inhibition of caspase-dependent initiation of apoptosis. TIAR is thought to be an effector molecule in apoptosis because it directly causes DNA fragmentation (Taupin, 1995), (Kawakami, 1992). Because its expression was decreased 1.68 fold, this is differential expression is indicative of a reduced likelihood of apoptotic triggering. Fas (aka CD95; confidence level 2, -2.52 fold change) is involved in CD8+ mediated T-cell cytotoxicity (Hanabuchi, 1994). Fas is the receptor

for Fas ligand (FASL). Fas-FasL binding activates the "death-inducing signal complex," and by proteolytic activation of caspase-8, initiates a caspase cascade which results in apoptosis (NCBI P25445). Thus, decreased expression of Fas by laser-treated cells is a strong indication of a reduced likelihood for apoptosis. JNKK1 (i.e. Jun kinase kinase 1; confidence level 2, -1.71 fold change) activates by phosphorylation of the kinase which in turn phosphorylates (Jun kinase) the dual purpose nuclear effector, Jun, which serves selectively as a transcription regulator or apoptotic effector (Mechta-Grigoriou, 2001). A reduction in JNKK1, therefore may serve to decrease both apoptotic signals and transcriptional rate of proteins necessary to cell proliferation, i.e. mitosis. p43/EMAPII (endothelial monocyte-activating polypeptide II; confidence level 1, -5.36 fold change) is involved in tRNA processing in its p43 form (Norcum, 2000), and when proteolytically cleaved the resultant EMAPII is a potent apoptotic cytokine that recruits monocytes to phagocytize the apoptotic cell remains (Knies, 1998). Similar to the dual purpose protein JNKK1 then, reduced expression of p43/EMAPII is indicative of both reduced protein translation and a reduced likelihood of apoptotic initiation. Peroxiredoxin V (confidence level 1, fold change equals +div/0 (not expressed in controls at all)) indicates a greatly increased expression. Overexpression of "[peroxiredoxin] inhibited p53-induced apoptosis" (Zhou, Y., 2000). Thus, increased peroxiredoxin expression correlates well with the other differential protein expression levels which are indicative of a reduced likelihood of apoptosis.

Smac/DIABLO is known to antagonize hILP if located in the cytoplasmic partition (NCBI NP_063940). Smac/Diablo is thought to carry the apoptotic signal from the cytoplasm to the nucleus by translocation. Exclusion of Smac/Diablo from cytoplasm

to the nucleus correlates with apoptosis (Verhagen, 2000). Cellular partitioning was a factor the assay used in this research cannot determine, though on the corroborative evidence of the proteins described above which show an anti-apoptotic effect, it is hypothesized that Smac/Diablo was located in the cytosolic compartment of the laser-treated cells. Bag-1 (confidence level 3) exhibits strong anti-apoptotic activity (NCBI Q99933). Its expression was reduced 1.71 fold. The regulation of Bag-1 activity is quite complex (Roth, 2001), but as a heat shock protein family member in the stress-induced cell it would function as an anti-apoptotic effector. Thus its decreased expression decreases inhibition of apoptosis. MST1 (confidence level 3, +1.74 fold change), functions as a positive apoptotic pathway feedback amplifying protein, (NCBI, Q13043), (Taylor, 1996), and showed increased expression. Thus increased expression of MST1 favors apoptotic signals via the JNK pathway and caspase cascades, (Ura, 2001), but the pathway is strongly inhibited downstream of MST1. In summary, the overall trend among apoptotic signalling proteins and apoptotic effectors indicates a strongly decreased physiologic readiness to trigger apoptosis involving multiple regulatory pathways.

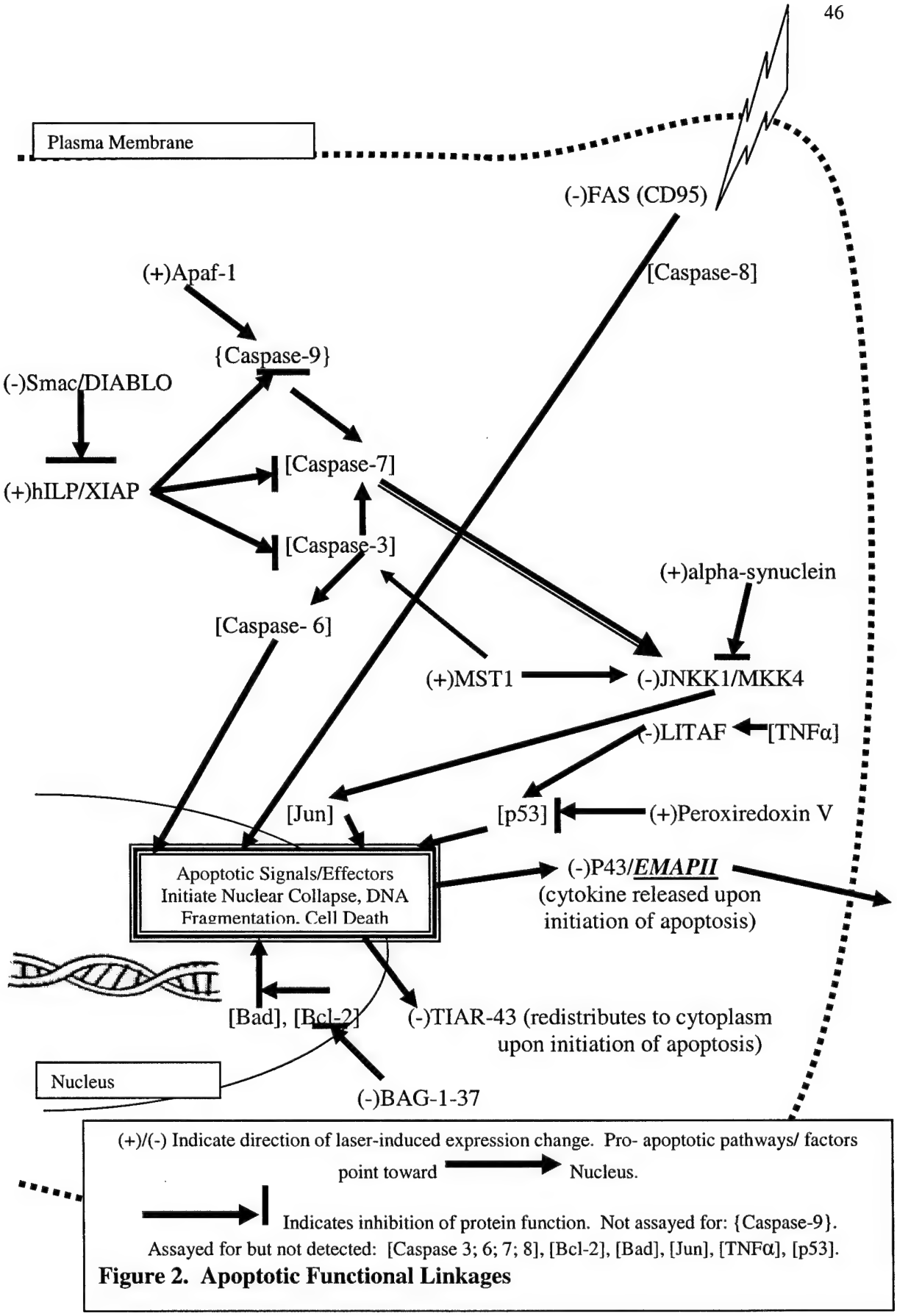


Figure 2. Apoptotic Functional Linkages

Transcription Factors

Eight proteins associated with DNA transcription were identified by the BD PowerBlot™ assay. The expression changes of all eight, with the possible exception of glucocorticoid receptor [which can signal either transactivation or transrepression, NCBI P04150], are indicative of a trend toward a decrease in transcription. La protein (confidence level 4) functions in the cell to protect nascent RNA polymerase (pol) III transcripts, i.e. tRNA, as well as to facilitate termination and reinitiation of transcription by pol III (NCBI P05455), (Fan, 1997), (Gottlieb, 1989). La showed a -1.71 fold change. FACTp140 (aka facilitator of chromatin transcription; confidence level 3, -2.01 fold change) regulates transcription by promoting access to the DNA in chromatin (NCBI AAD43978), (Orphanides, 1999). TATA binding proteins (TBP) are essential for assembly of eukaryotic transcription complexes (Weaver, 1999). In conjunction with TBPs, TAF-172 (TBF-associated factor, 172 kDa, confidence level 3, -1.79 fold change) regulates transcription by promoting assembly of RNA polymerases I-III. Decreased TAF-172 expression means fewer assembled RNA polymerases (NCBI O14981), (Chicca, 1998). Based on their putative functions the cumulative effect of their respective (-2.01 and -1.79) fold changes indicate that DNA polymerases would be less likely to assemble and those that have assembled would have reduced access to DNA. Glucocorticoid receptor (confidence level 2, fold change +1.37) can function in transactivation or in repression (NCBI P04150), (Leclerc, 1991), (Reichardt, 1998). The function it performs at any given time cannot be determined by the expression assay performed in this research, though based on the differential expression profiles of the other transcription factors, it is hypothesized that glucocorticoid receptor pathways were

functioning to reduce transcription at the time of cell harvest. The remaining three transcription factors depicted in Appendix C show expression patterns that correlate well with those proteins described above whose differential expression patterns suggest decreased transcription. p54nrb-56, a DNA transcription factor (NCBI Q15233), (Dong, 1993) and p54nrb52 which activates topoisomerase I (NCBI Q15233), (Straub, 1998), (Basu, 1997) both show decreased abundance. Topoisomerase I functions to relieve the torsional stress that occurs when DNA is unwound by creating transient nicks in the DNA, so topoisomerase activation is important during transcription and replication (Weaver, 1999). CtBP1-41 is a corepressor for transcription factors (NCBI Q13363), (Sewalt, 1999), so its +2.90 fold increase also correlates with the cumulative trend indicative of decreased transcriptional activity. In summary, it appears that transcription is generally shut down as indicated by reduced access to DNA in chromatin, reduced RNA pol I, II, and III formation, and decreased topoisomerase activity. There also appears to be reduced tendency for pol III (tRNA) transcription activity due to reduced expression of La protein.

Translation Factors

Six translation factors showed an even split between trends toward increased and decreased expression, giving the appearance of a mixed response. eIF-5a (confidence level 5, +7.57 fold change) is a eukaryotic translation initiation factor that purportedly has multiple functions including nuclear export of proteins. eIF-5a has a well recognized functional role in the association of the ribosomal 48S and 60S subunits to form a functional ribosome ready for translation of RNA to protein (Weaver, 1999).

Additionally, it is postulated that nuclear export may be the major functional role of this protein and initiation of translation may be a minor role (Hofmann, 2001). If nuclear transport is a major function for eIF-5a, its significant up regulation may be an indication of nuclear export of damaged proteins to the cytoplasm for degradation ("molecular debridement"). eIF-5a showed an average up regulation of nearly eight-fold and so shows initial promise as a candidate to be a laser-tissue interaction biomarker. Expression of two additional proteins was upregulated. p70s6k, which phosphorylates ribosomal protein S6, thereby stimulation translation, (NCBI P23443), (Grove, 1991), and AKAP149 which may function in the phosphorylation-dependent regulation of all RNA processing (Trendelenburg, 1996) both showed signs of significant up regulation (+2.07; +3.55), though their confidence levels were low. See appendix C.

Three proteins showed an expression trend which is potentially indicative of decreased translation. TRAX (confidence level 3, -1.76 fold change) plays a role in the regulation of mRNA transport and translation (NCBI NP_005990), (Aoki, 1997). p54nrb-56 (confidence level 2, -2.57 fold change) functions in pre-mRNA splicing, (NCBI Q15233), (Dong, 1993). p43 (confidence level 1, -5.36 fold change) complexes with an aminoacyl-tRNA synthetase (Norcum, 2000), (Kao, 1994). The trend with these three proteins seems to indicate a reduction in translation of polypeptides. But there are strong indications of a rebuilding of the translational apparatus see Vesicular Trafficking, below. Based on the observation that the laser-treated cells eventually recover and thrive, it is likely that the reduction in these proteins is a temporal event that remains in effect until the cell has time to recover from damage incurred as a result of laser-treatment.

Vesicular Trafficking

Differential protein expression fold changes were noted in eleven proteins which function in vesicular trafficking, i.e. the control and movement of endo- and exocytotic vesicles, as well as vesicles that transport proteins between the endoplasmic reticulum and the Golgi apparatus or the nuclear membrane for example. Of these eleven there were four showing decreased in expression and seven showing increased expression, see Appendix C.

A trend of increased expression of intracellular vesicular trafficking proteins emerged. BiP/GRP78 (binding protein, confidence level 3, +3.05 fold change) is a chaperone protein localized to the lumen of the endoplasmic reticulum (ER), which receives newly translated proteins for modification. BiP/GRP78 functions to chaperone unassembled secretory proteins as well as to assemble multimeric protein complexes inside the ER (NCBI P11021) (Linnick, 1998). GS27 (Golgi Snare of 27 kDa, confidence level 3, +2.75 fold change), is a vesicular transport protein localized to, and functioning in, transport from the cis (inside face) of the Golgi apparatus to the trans (plasma-membrane side) of the Golgi (Lowe, 1997). ARF-3 (ADP-ribosylation factor-3, confidence level 3, +1.37 fold change) exhibits lipase activity and also regulates traffic through the trans Golgi network (TGN) (Boman, 2000). The Golgi itself receives proteins from the ER for post translational modification and assists in their transfer by vesicular budding (Lowe, 1997). Bet1 (confidence level 1, +3.73 fold change) is implicated in unidirectional trafficking from the ER to the Golgi (NCBI NP_005859), (Hay, 1996). The increased expression of these vesicular sorting and transporting proteins suggests the cell may be poising itself for increased translation by preparing the

compartmentalized machinery that is necessary for processing those proteins prior to actually beginning their translation.

Four proteins showed decreased expression. Neurotensin receptor 3 (confidence level 4, -2.01 fold change) is a neuropeptide receptor that is localized in the Golgi compartment and vesicles (NCBI NP_002950) and also to the plasma membrane (Nielsen, 1999). It functions in the sorting of proteins to be transported in vesicles and also in the endocytosis and degradation of lipoprotein lipase. Caveolin 1 (confidence level 2, -1.79 fold change) is a scaffold protein (it functions as a framework that other proteins/lipids bind to) that is essential to formation of membrane invaginations called caveolae (NCBI NP_001744). It functions in vesicular transport and cycles between the plasma membrane and Golgi in a microtubule-dependent fashion (Wary, 1998), (Conrad, 1995). Rab 11 (confidence level 2, -1.51 fold change) is a small GTPase that helps control traffic through pericentriolar (recycling) endosomes (NCBI CAA40064), (Ullrich, 1996). Rab 8 (confidence level 2, -3.25 fold change) is a related GTPase that functions in regulation of vesicle transport from the TGN to the plasma membrane in epithelial cells (NCBI CAA40065), (Peranen, 1996). It is interesting to note that expression of these proteins which are involved with transport to-and from the plasma membrane was down regulated, while expression of vesicular transport proteins localized to the ER and cis/trans Golgi network was up regulated. This observation corroborates the hypothesis of a molecular debridement phase of recovery in that a damaged cell would likely commit less energy to the translation of proteins destined for exocytosis during the period of debridement and subsequent recovery when it would need to reestablish its normal

complement of metabolic machinery which sustained damage (a reconstruction phase) related to the affects of a xenostressor (impingement of laser light in this case).

Complexin 2 (confidence level 2, fold change = +div/0) was expressed in laser-exposed cells, but not in controls. Complexin 2 is believed to function in regulation the protein-protein interactions that occur during fusion of exocytotic vessels with the plasma membrane (NCBI O42105), (McMahon, 1995). Increased exocytosis may indicate an attempt by the cell to rid itself of photochemical byproducts, damaged proteins, toxins, etc., in a process akin to dumping un-recyclable trash. p47A (confidence level 2, + 1.47 fold change) complexes with other proteins to form a multimeric adaptor protein complex (AP-3) that functions in the sorting of integral membrane proteins as they are trafficked between the TGN and destination membranes (Dell'Angelica, 1997). Importantly, the AP-3 complex with which p47A associates with has been implicated by genetic studies to play a role in the sorting of proteins to lysosomes where controlled digestion of macromolecules occurs. Thus the upregulated p47A is involved not only in vesicular transport, but also in protein degradation.

Cell Adhesion and Motility

Eleven distinct proteins associated with cell adhesion and motility were identified. Observed differential expression trends indicated decreased adhesion, motility and adhesion-based signalling. ROCK-I (confidence level 3, -2.82 fold change) is involved in the regulation of focal adhesion through formation of stress fibers (Ishizaki, 1996), (Tominaga, 1998). Reduced expression indicates a reduced tendency to adhere to surrounding cells and cellular substrate.

Ral A (confidence level 3, -1.70 fold change) is a GTP-binding protein that functions as an intermediate in a signalling pathway transducing the epidermal growth factor signal, and ultimately results in increased cell motility (NCBI TVHUAA), (Gildea, 2002). This effect is implicated in the invasiveness and tendency of tumor cells to metastasize (Gildea, 2002). Thus, decreased expression of Ral A implies decreased cell motility.

Suppression of RECK (confidence level 3, + 1.76 fold change) is thought to be required for both tumor cell invasion and metastasis (Takahashi, 1998). The observed increase in RECK expression is another indicator of a trend toward decreased cellular motility, a characteristic deemed essential to the processes of invasion and metastasis. Interestingly, though Takahashi reports (1998) that RECK "is undetectable in tumor-derived cell lines and oncogenically transformed cells," in our experiment, RECK was detected both in control and laser-exposed cells.

FAK (focal adhesion kinase, confidence level 2, +1.99 fold change) colocalizes with integrins (leukocyte-epithelial cell adhesion factors) and is also thought to regulate cell motility, and adhesion-dependent survival (NCBI Q05397). Adhesion triggers intracellular signalling cascades and so FAK is thought to mediate integrin signalling cascades (Hildebrand, 1993). Increased expression of this kinase may indicate an increase in some adhesion-mediated signalling pathways.

Ninjurin-18 (confidence level 4, + 1.78 fold change) is a mediator of adhesion expressed predominantly in epithelial tissues whose expression is induced by nerve injury, promoting axonal growth (NCBI NP_004139), (Araki, 1996). Increased expression may signal initiation of a repair mechanism. Gephyrin (confidence level 3,

+1.75 fold change) is thought to function in postsynaptic neurons as a microtubule anchoring protein (intracellular structural protein) linking glycine receptor to the cytoskeleton (NCBI NP_065857) (Prior, 1992). Glycine is an inhibitory neurotransmitter in glycinergic synapse (Legendre, 2001). Contactin (confidence level 2, -4.88 fold change) "is a neuronal cell adhesion molecule" which may function in the formation of new axon connections in the nervous system. It is interesting to note the expression of proteins usually found in neuronal cell lines being expressed in the epithelial cells (A2058, malignant melanocytes) used in this experiment. Ninjurin, Gephyrin, Contactin, and Dystrobrevin-84 fit into this category and are all involved in the formation or repair of nerves. It is possible that these proteins are expressed in malignant melanocytes due to both the misregulation of gene expression characteristic of cancers that often results in de-differentiation, or due to the embryonic origin of melanocytes as cells arising from the neural crest (Montagna, 1956), and so might be expected.

CD54/ICAM-1 (intracellular adhesion molecule, confidence level 4, +1.73 fold change), a pleiotropic protein with both immunologic and cell-cell/cell-matrix adhesion functions, showed increased expression and is discussed more fully in the section titled "Immunology," below.

Three proteins involved in actin processing interactions showed expression changes indicating actin fibers formation and maintenance will slow or halt. Stathmin/Metablastin (confidence level, fold change = + div/0) was not detected in the control samples, but was detected in laser-treated cells. Stathmin functions in the control of microtubule formation. Active Stathmin destabilizes microtubules by preventing assembly and promoting their disassembly, (NCBI P16949), (Brattsand, 1994) i.e.

"molecular debridement." The up regulation of Stathmin seen in the laser exposed cells indicates actin fibers will tend to dissemble and not be reassembled in the cell.

Demantin-61 (confidence level 3, -1.50 fold change) and Demantin -57 (confidence level 2, -2.36 fold change) are isoforms of the same erythroid actin bundling protein (NCBI I39062), (Azim, 1995). These proteins function in the membrane skeleton (i.e. they help membranes maintain their shape). Assuming Stathmin translation is coordinated with reduced expression of Demantin, reduced demantin expression makes sense as there will likely be less actin to bundle due to the action of Stathmin. GelsolinG37820 (confidence level 1, - 16.05 fold change, see Appendix B) is an actin-modulating protein that severs actin filaments in a Ca^{2+}-dependent manner (NCBI 1211330A), (Kwiatkowski, 1986). Actin severing is an important step in the events leading to a change of cellular shape (Bearer, 2000). This observation correlates with the fact that with less actin in the cell due to the action of Stathmin, it is energetically sound for the cell to reduce Gelsolin production. Actin filament severing may represent another aspect of molecular debridement as well.

Thus the trends observed in proteins associated with cell adhesion and motility indicated reduced cellular adhesion, motility and adhesion-based signalling, and a possible molecular debridement component.

Immunology

Six proteins directly associated with immune system functions were identified. Of these, three showed increased fold changes and three showed decreased fold changes.

Apparent functional trends include cellular preparation to participate in an inflammatory immune response, and decreased expression of apoptotic factors.

LITAF and Fas/CD95 both showed trends of decreased expression, indicating a reduced readiness to initiate apoptosis. See discussion of LITAF and Fas/CD95 above, "Apoptotic Factors," for a detailed discussion.

CD54/ICAM-1 (intracellular adhesion molecule, confidence level 4, +1.73 fold change) is an integrin ligand (discussed below) and is also the major receptor used by rhinoviruses to gain entry to the cell (NCBI P05362), (Greve, 1989). ICAM-1 mediates adhesion of leukocytes to endothelial cells, which is critical to the inflammatory process. Increased ICAM-1 expression may be an indication that the laser-treated cells suffered damage and are preparing to participate in an immune response to the damage, in this case by expressing a surface antigen that will allow phagocytic monocytes to bind to their cell surface. This effect would likely be of value to the human organism as a whole if the cell's physiologic 'decision' was to execute the apoptotic program. Integrin beta-1 (confidence level 1, fold change = (-) div/0) normally functions as a leukocyte-epithelial cell adhesion factor (NCBI P05556). Integrin beta-1 is the ligand for CD54/ICAM-1, and also functions as a cell-cell or cell-matrix adhesion molecule. The down regulation of integrin beta-1 would result in a decrease in cell adhesion both to neighboring cells and to the matrix upon which the cells are growing. This down regulation may also reflect the increased internal energetic requirements of dealing with molecular debridement and demolition/rebuilding phases of stress response that would decrease availability of cellular energy resources for other purposes such as interacting with the environment external to the cell.

GAGE (confidence level 4, +1.89 fold change) is a commonly expressed melanoma surface antigen (NCBI AAA82744), (Van den Eynde, 1995). Thus, GAGE expression by A2058 cells is to be expected, as they are a melanoma cell line. Though melanomas typically express GAGE, they nonetheless are able to evade surveillance and destruction by cytotoxic T lymphocytes. I hypothesize that GAGE expression may signal to cytotoxic T lymphocytes that the cell is not to be killed, therefore increased GAGE expression may indicate a further attempt to protect the cell from executing an apoptotic program. GAGE expression involves a demethylation transcription control mechanism, so increased expression is evidence of increased DNA demethylation in laser treated cells as previously reported by De Backer, (1999). Tyk2 (confidence level 3, +1.51 fold change) is a widely expressed tyrosine kinase that induces transcription of genes involved in the response to interferon alpha and beta (NCBI TVHUY2), (Velazquez, 1992), (Watling, 1993). Increased expression of this gene correlates with the observed trend of preparation to deal with an immune response discussed above in conjunction with CD54/ICAM-1 expression changes.

DNA Repair Proteins

DNA repair-related protein expression is of interest because DNA damage by laser-tissue interaction was hypothesized. The BD PowerBlot™ assay identified only two proteins with a direct role in DNA repair attributed to them, and both showed negative fold changes, indicating there was a trend toward reduced DNA repair. MSH6 (confidence level 3, -2.57 fold change) when complexed with another protein, MSH2, binds to DNA containing guanine/thymine mismatches owing to its ability to detect

single pair mismatches as well as insertion/deletion loops (Gradia, 1997). XPA functions in nucleotide excision repair (NCBI JG0190), (Aboussekhra, 1995), (Evans, 1997). Expression of XPA was down regulated 2.95 fold (confidence level 1). Due to the apparent general decrease in mitotic activity in the laser-exposed cells, it is hard to say whether DNA repair appears reduced due to an actual lack of damage, or if the cell has not yet recovered from the molecular perturbations due to laser irradiation that it can mount an effective repair effort if needed. It could be that a transcriptional response is being mounted, but not yet detected by the protein expression assay employed in this research at the 24-hour post exposure harvest time. It is noteworthy that Rac1 (confidence level 1, + div/0 fold change) which functions in DNA repair via a Ras/PI3K/Rac1/NADPH oxidase-dependent pathway (NCBI TVHUC) (Cho, 2002) was upregulated, as was PI3K (confidence level 1, + 2.98 fold change). The DNA repair response, if necessary, might be seen at a later time in the laser treated cells. Lack of evidence of DNA repair does not mean there is no DNA damage, especially as it has been shown to be a result in 532nm lased cells (Cao, 1993). Nonetheless, mediators of DNA repair do not appear to be promising laser-tissue interaction biomarkers at the 24 hour endpoint based on these findings.

Miscellaneous Functions

Seven proteins were detected whose functions did not fit into the above categories. Some of these, like Na,K ATPase beta are constitutively expressed, in this case as a Na+ and K+ ion pumping protein (NCBI AAB61713), (Stengelin, 1997).

Others are disease associated, such as HAP-1, a Huntington disease-associated protein (NCBI S67493), (Li, 1995).

Two particularly tantalizing proteins are mEPHX (confidence level 2, -1.56 fold change) and p62 Ick ligand (confidence level 1, +4.46 fold change). mEPHX functions in reactive epoxide metabolism as a hydrolase enzyme critical to the detoxification of cellular toxins (NCBI A29939), (Hassett, 1994). Based on the expected increase in production of reactive oxygen species in the course of the cellular response to laser damage, it is reasonable to expect that mEPHX expression would increase rather than decrease because of the role mEPHX plays in reactive epoxide metabolism. It would be interesting to do a horizontal study of the expression changes of mEPHX over time. p62 Ick ligand is implicated in binding the SH2 domain of Ick, a protein whose function I was unable to elucidate from the literature. Ick is known to be constitutively associated with CD44, and CD44 binding is associated with both cell proliferation and apoptosis (Foger, 2000). Research into p62 Ick ligand, and Ick function remain items of interest then due to the opposing proliferation/apoptosis signalling p62 Ick ligand may be involved in.

Function Unknown

Many of the proteins detected were pleiotropic, i.e. they have more than one known function. In contrast, one protein, drp1 stands out because its function is currently unknown. Drp1(confidence level 5, -2.68 fold change) is known to be regulated in a density dependent manner, its expression being greatly increased in high density cell cultures as compared to normal tissues. Expression is also independent of serum starvation effects (Deyo, 1998). As it was observed that cellular adhesion and adhesion-

based signaling were decreased in the laser-treated cells, I hypothesize that some aspect of the perturbation caused by laser treatment interrupted the normal intercellular contact-dependent signalling necessary for high levels of drp1 expression .

Conclusions

Human melanocytes grown in tissue culture were exposed to 532 nm picosecond pulsed laser light (light green), and were harvested and lysed at a 24-hour endpoint. The cellular lysate was subjected to gel electrophoresis, Western blotted, and probed by immunologic assay to detect differences in protein expression of 859 different proteins. A major goal of the research was to find protein candidates for use as laser-tissue interaction biomarkers and understanding of the physiology of lased tissues at the cellular and molecular levels. Notable differences in protein expression between experimental and control group data sets (see Discussion above, and Appendices B and C) suggest that the hypothesis is correct, i.e. Exposure of human melanocytes grown in tissue culture and exposed to high-energy, ultrashort pulse, laser energy resulted in genetic induction measurable by protein expression as determined by immunologic screening of western blotted of protein samples.

One goal of the research was to identify potential laser-tissue interaction biomarkers. Ubiquitin-conjugating enzymes Ubc9 and UbcH7 (cellular markers of proteins to be proteolytically degraded by the cell) generated high confidence signals with high expression fold increases via western blot immunologic assay, making them good candidates as laser-tissue interaction biomarkers. Additionally, eIF-5a, a eukaryotic

translation initiation factor, and BiP (binding protein), (a chaperone molecule), shows promise as laser-tissue interaction biomarkers. Based on their differential protein expression profiles, Sam68, P160, and hILP have potential to serve as biomarkers for laser exposure as well.

Another major goal of the research was to characterize the physiological state of the cell following laser treatment. Differential protein expression trends were observed which indicate a significant down regulation of mitosis (cell cycling), apoptosis, transcription and translation, actin fiber formation and maintenance, and DNA repair. At the same time, it appears that protein degradation pathways were up regulated, as were intracellular vesicular trafficking and signalling to marshal and/or respond to an inflammatory immune response.

In an attempt to foster a general understanding the cellular response to the physiologic challenge posed by laser-light and the kinetics involved I offer the following paradigm of stress response and recovery. The paradigm was developed in the course of discussion with Dr. John Obringer, and credit for developing the paradigm is shared with him (see Figure 3, Proposed Cellular Stress Response and Recovery Paradigm, below). This research describes cells at the 24-hour point post-exposure to the laser-light stressor, indicated on Figure 3 by the dashed vertical line. This paradigm is intended to be descriptive for sub-lethally exposed cells, but not to cells that were not frankly ablated or directly killed by the effects of laser treatment.

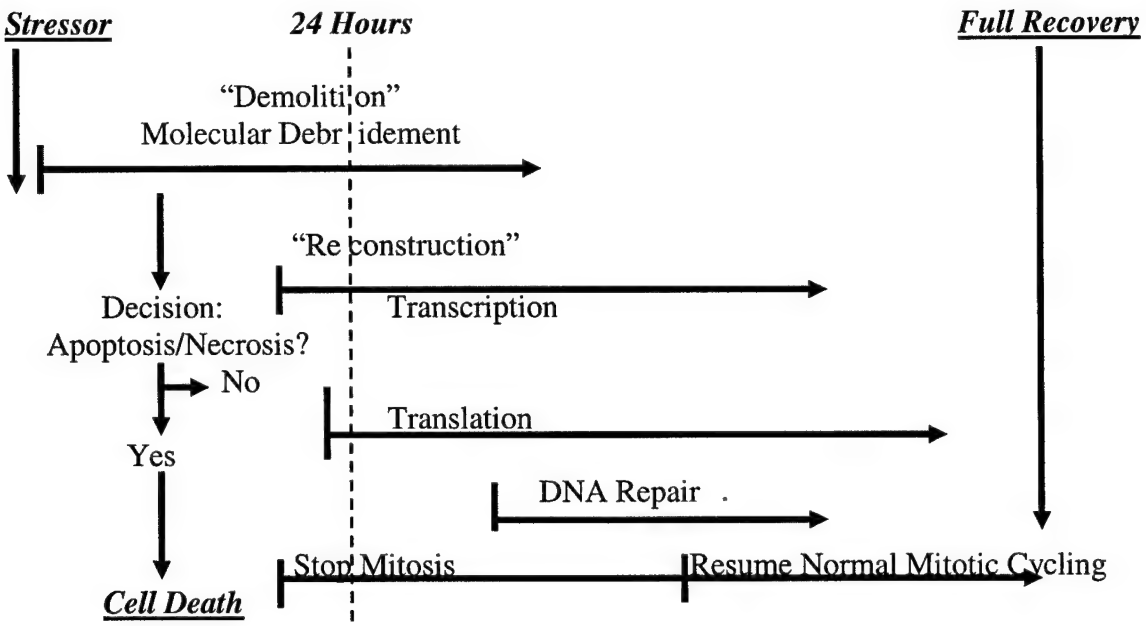


Figure 3. Proposed Cellular Stress Response and Recovery Paradigm.

In conclusion, it appears that though humans are not specifically adapted to withstand laser insult (a xeno-stressor), within the human genome there exist sufficiently flexible preadaptations relevant to other stressors such as ultraviolet radiation and oxidative stresses to enable individual cells to survive the unexpected perturbations caused by laser treatment. The resiliency of the cellular response when dealing with such an unexpected perturbation is noteworthy, and parallels the response of individual organisms, populations, communities, and indeed each level of hierarchy of biological organization above the cell. Much work remains to be done in assessing the nature of the cellular response to laser light. Reduced mitotic cycling and decreased tendency to initiate apoptosis appear to be two such responses. It will quickly be understood by persons engaging in this endeavor that the essence of this work hinges on understanding the regulation and expression of the genetic program which makes possible this profound resiliency to perturbation rather than focusing on any single protein, pathway, or process.

Recommendations for Further Study

In addition to the single 24-hour endpoint of this experiment it would desirable to characterize the response of cells to laser exposure of at a number of different time points, such as 1, 3, 6, 9, 12, 18, and 48 hours, etc., in order to track the changes in protein levels over time until full cellular recovery, in an attempt to better understand the differential expression changes as the cell adjusts to the perturbation it received from the laser treatment.

There are a number of laser parameters that can be varied and would be expected to have a large impact on the cellular response. For example, this research used 20 separate 120 picosecond 532 nm pulses with peak energies of 45 (+/- 3) mJ per pulse yielding a total fluence per well of 1.9 Joules. To more fully characterize the effects of laser-tissue interactions at the cellular level it will be necessary to perform a number of different experiments with different wavelengths of light, varying power levels, numbers of pulses, total energies, and pulse widths.

As noted earlier, the experimental cell line, A2058 was from a human melanoma. It would be desirable to use a non-cancerous cell line for further research to avoid the confounding influence of abnormal genetic expression patterns displayed by cancerous cells.

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Note: References not cited in the text are cited in Appendix C, Protein Function Table.

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BD Catalog #	MW	Protein ID	BD Catalog #	MW	Protein ID
A58920	220	AKAP220	A50620	22	ALG-2
A59420	125	Amphiphysin	S55820	150	Symplekin
V39620	97	VCP	P76420	113	PARP
G73320	78	BiP/GRP78	S70720	89	Stat4
C26920	61	Calcineurin	S60720	76	SLP-76
C40520	47	Casein Kinase I epsilon	C86720	55	p55Cdc
C69520	29	Calretinin	T50320	34	TRADD
A60520	182	AF6	G16720	24	GRB2
C38020	100	R-Cadherin	M62420	357	Mitosin
C46920	68	CaM Kinase Kinase	L65320	180	Integrin alpha L/LFA-1
C20020	53	CDC42GAP	C40320	120	c-Cbl
P46220	36	p36	C62920	76	CUL-2
C73220	23	CRP1	I71320	46	IAK1
A41820	180	AP180	C18520	33	Cdk2
C63120	120	CASK	C70820	22	CDC42
G72120	84	GOK/Stim1	D80020	160	DSIF
M32320	68	Munc-18	R56420	110	rSec8
N35120	47	Neuronal Pentraxin	A30120	70	Annexin VI
R35520	25	Rab3	P71720	55	PDI
C60320	19	Complexin 2	H65220	35	HAX-1
M98820	219	MUPP1	C74720	150	CA150
N68720	145	beta-NAP	D38220	103	DP103/Gemin3
P93120	120	PACS-1a	D63020	87	Dystrobrevin
E18220	97	eps8	Z24820	70	ZAP70 Kinase
C49020	58	beta 1-Calcium Channel	F22120	45	Fas/CD95/APO-1
B33720	47	BAF47	E78920	27	ERAB
N63520	23	Neurogenin 3	A79920	160	AIB-1
N33320	220	Nogo-A	A78420	95	HIF-1 beta/ARNT1
B58720	125	BM28	C28420	60	CaM Kinase IV
G60420	100	Gap1m	P98120	42	p38 alpha/SAPK2a
D62320	87	Dystrobrevin	R23520	24	Ral A
R68520	25	Rab4	R33220	130	RPTP alpha
S63320	19	alpha-Synuclein	A36120	104	Adaptin gamma
T33520	50/42	TIAR	G81020	68	G3BP
C57020	150	CD100	F65020	48	Flotillin-1
D37920	117	DGK iota	P24620	23	PMF-1
P36520	78	PKC delta	R75020	154	Rad50
T60820	60	Tpl-2	N58820	120	NFAT-1
B46620	26	Bcl-2	H76520	76	HEC
D80320	84/79	DLP1	N88520	60	p54nrb
T57120	50–68	Tau	E59020	45	ERP
N31020	155	nNOS/NOS Type I	L73120	32	LAIR-1
P18420	115	p115	G10020	210	GMAP-210
S35020	25	SNAP-25	F39620	140	FACTp140
S55720	69/55	ShcC	H86320	66	hPrp17
M58320	140/88/80	Mena	R92420	36	RNase HI
C81820	115	CLIP-115	M82020	19	Stathmin/Metablastin
M66420	44	JNKK1/MKK4	N91820	280	NPAT
R56220	21	Rac1	T11520	140	TTF-I
P60220	10	PIN	S83720	106	SATB1
M41420	280	MAP2B	T84620	66	TRF2
E64320	145	eps15	S86420	55	SKAP55
P43520	95	PSD-95	P39520	42	p38 alpha/SAPK2a
R43020	58	Karyopherin alpha 2	E80720	27	eIF-6
G51820	40	G alpha t	C93420	240	CRIK

G54220	23	GCAP-1	E95720	110	Exportin-t
R69420	173	Rim	S95620	74	SNX1
G74820	111	GluR delta 2	M96020	38	GADS/Mona
P14820	90	PKC epsilon	A14120	11	Annexin II Light Chain
Y35320	62	Yes	A97420	450	AKAP450
V10720	45	Vesl-1L	A93520	150	Ataxin-2
R28720	25	Rab5	P17320	68	PTP1C/SHP1
T16020	145/95	trk B	A37720	50	Arp3
S39520	65	Synaptotagmin	J12420	38	JAB1
G45020	45	Glutamine Synthetase	556433		Cytochrome c
S52520	29	Synaptogyrin	S93920	340	SMRT
A63220	20	ARF-3	P94520	110	PI3-Kinase p110 alpha
G16920	91/84	Stat1 (N-terminus)	T93820	65	TLS
P68620	50/47	p47A	T92520	33	TRAX
N38120	180	NMDAR2B	B10520	150	Btf
S41820	130	EphA4/Sek	P14420	110	PI4-Kinase beta
R52320	25	Rab27	B11020	60	BAF60a
N73720	120-150	Neuroglycan C	H22020	40	Hsp40
C96420	95	cGB-PDE	S13120	35/36	alpha-/beta-SNAP
S56820	74	Synapsin IIa	T10620	161	TopBP1
M54920	49	pan-JNK/SAPK1	D20320	103	DNA Ligase III
C44920	22	CBF beta	Y24420	74	YAP
P69120	50/47	p47A	A61220	56	Annexin XI
S63820	290/280	SSeCKS	E15920	18	eIF-5a
D70920	110	DGK theta	E17120	42–85	pan ERK
M61420	84	ADAM9/MDC9	S15520	170	Sos1
S71120	60	Smad4/DPC4	S66020	110	Striatin
A14020	36	Annexin II	C18720	33	Cdk4
B67020	96/89	BRAMP2	C38320	18	Caveolin 3
J55020	180/160	Tenascin-R/Janusin	U68920	400	Utrophin
F72520	265	Fatty Acid Synthase	G70220	160	MSH6
S66220	58	Smad2/3	C56520	80	L-Caldesmon
E39120	38	EB3	B54120	56	B56 alpha
T69020	85-95	TGN38	R24720	21	RBP
M74420	196	panMunc13	P66520	140	p140mDia
R94920	190	Roaz	M34520	102	MSH2
C13720	43	Connexin-43	F69620	75	FIN13
P62620	25	PCMT-II	G22320	46	GSK-3 beta
F64420	14	FKBP12	C12720	34	Cdk1/Cdc2
K96920	80/85	KIF3A	C37120	22	Caveolin 1
P71020	129-133	PMCA2	M68420	150	mSin3A
M86920	52	JNKK2/MKK7	G51620	93	Gephyrin
R38620	23	Rab24	C22420	70	Cox-2
I71920	273	IGF2R	E34620	51	Ets-1
C75120	125	Integrin alpha v	P64720	82	PKC eta
P17720	80	PKC beta	T39720	37	TBP
E16820	66	EAAT2	T85720	23	Tim23
P19920	48	PKA RI	T82220	200	Thrombospondin-2
C38720	19	Cofilin	C37020	120	E-Cadherin
M72620	133	mGluR1	X98220	87	XPD
S74920	90	SIRP alpha 1	E16320	62	ERK3
M79820	71	MEKK3	C77720	45	Cab45
D74620	50	Dynactin	N95520	110	Nedd4
D97520	32	DARPP-32	F82720	85	FLAP
N79020	180	Neurabin	M93620	61	Mint3
S96320	100	SHPS-1	A78720	41	AIM-1
N80220	80	Numb	G83820	28	GS28

E85120	66	ENC-1	K99720	115	KSR-1
B40820	42	B2 Bradykinin Receptor	P10420	90	PEX5
G55620	45–60	GDNFR-alpha	H98620	40	HspBP1
R81520	160	ROCK-I/ROK beta	K98220	95	KIF3B
T89420	130	Tomosyn	A64120	38	AMPK beta
H69220	92	HNF-1	B12520	19	Bog
V76720	135	Integrin alpha 3/VLA-3	S68020	66/52/46	SHC
C26220	82	gamma-Catenin	T66920	240/230	TLP1
O79120	65	Occludin	F97320	80	4F2 hc/CD98HC
P53620	49	PKA RI alpha	R61720	29	RanBP1
H28520	59/56	Hck	R66120	135	Ras-GRF2
G25020	250	LR11/SorLA/gp250	I16620	95	Insulin Receptor beta
S12520	140	Synaptojanin 1	P61520	62	Dok1/p62dok
R44520	75	Rabphilin-3A	P33720	47	p47phox
M64620	35	Caspase-7/MCH-3	N13920	40	NUDT5
R81320	245	RAFT1/FRAP	L59920	220	Laminin B2
M75920	120	Mint1	A96220	150	AOX1
A80820	100	ABR	V34420	95	Villin
P75520	58	PP5/PPT	E77020	49	eIF-5
P35220	36	PP1	H59320	32	Heme Oxygenase 1
E99620	140/200	ESE-1	D77620	48-52	Dematin
A22220	110	AFAP	T77420	172	TAF-172
C61820	50	Carboxypeptidase E	A74220	95	AKAP95
L80120	42	LAT	M99920	25	MnSOD
C40420	25	Casein Kinase II beta	S10520	17	Spot 14
D67820	140	Dlg	S86120	34/37	SCP3
P47920	68	PLK-1	P75220	110	PI3-K p110 delta
N69920	45	Na,K ATPase beta 2	P69820	67	p67phox
C76920	32	Caspase-3/CPP32	N77920	52	NDP52
P82120	87/90	PIP5K gamma	I94320	180	IRS-1
C70320	130	N-Cadherin	L78220	55	LAP1
P18020	50	PTP1B	P77820	45	p45/SUG1
A81420	105-130	AKAP-KL	C81120	29	Cellugyrin
P67920	59	PKB alpha/Akt	S59220	57/45	Selenoprotein P
T38020	49	TCBP49	I84520	113	Itch
P11920	24	p24	S87620	70	p70s6k
M38420	130	MYPT1	U85520	21	UbcH6
F84020	105	Frabin	M17520	83	MCM
E86620	80	Endopeptidase 3.4.24.16	L86020	60	Lysophospholipase
H82420	50	Hic-5	B10020	43	BPntase
P83620	36	Psme3	M30220	97	MTP
S88220	22	sigma 3A	M35020	42	alpha-Methylacyl-CoA Racemase
K87020	125	K Channel alpha	B32320	18	Bet1
P82820	80	PKC gamma	F14420	240	Fibronectin
V83320	45	Vesl-1L	R78620	117	Rabaptin-5
V85620	29	Vti1a	S65720	62	STI1
R79220	125/94	RNCAM	M24520	46	MEK2
M11220	214	Myosin Vb/Myr6	R32620	25	Ran
M10020	190	MRCK alpha	E19420	185	erbB2
P70520	150	phospholipase C beta 1	C24120	120	P-Cadherin
P24120	45	PhLP	G37820	93	Gelsolin
H89720	85/98	HAP1	P62220	68	PKR
C14020	48/96	CPG16/CaM Kinase VI	P47720	36	PP2A Catalytic alpha
P76820	170	PI3-Kinase p170	B73520	21	Bax
S80620	115	SRPK2	G12920	120	Ras-GAP
G87120	88/90	GSPT2	R23820	90	Rsk
B68820	145	Brevican	H75420	58	hHR23B

P90620	48	PP2C delta	T41520	33	TFIIB
N99820	21	NCS-1	M51920	26	Mxi-1
C79620	130	M-Cadherin	N42420	15	NTF2
P81620	105	Per2	P77520	110	PNUTS
F10020	75	FEZ1	P13020	85	PI3-Kinase
A73820	49	Acetylcholine Receptor alpha	T70420	53	TEF-1
C98320	160	delta-Catenin	M17020	45	MEK1
G90520	130	GRIP	C28620	33	Cyclin D3
N98720	97	Nexilin	R02120	21	Ras
C10220	62	N-Copine	I61620	100	IRAK
A59120	35	Arginase I	A27320	68	Acetylcholinesterase
Z98920	160	ZO-2	P72020	42	p38 delta/SAPK4
G12020	130	GABA B R2	R60020	25	Rab5
C25520	89	CUL-3	S61920	15	SIII p15
S91920	39	STRAP	S21120	91/84	Stat1 (C-terminus)
Cabc20		CaM Kinase II b	P12220	148	Phospholipase C gamma
T93020	116	TAO1	L05620	56	Lyn
G94420	95	GIT1	S55920	40	SMN
A92120	50	AP50	R23020	21	Rap2
C16120	24/28	COMT	L33420	150	LAR
N38920	140	Neurabin II	T57720	101	Transportin
P94020	95	p95PKL	H62120	57	hILP/XIAP
T34120	64	Tubby	D56620	46	DAP3
A34020	130	ASAP1	F36620	24	FADD
A11120	59	APP-BP1	P13320	85/80	Cortactin
P34620	15	Peroxiredoxin V	C67120	150	CAF-1 p150
I55220	150	Integrin alpha 5	G51720	61	GRB14
L44820	110	LRP	D71820	43	DEK
H38220	90	Hsp90	H42020	23	HRF
P20520	74	PKC iota	C64020	43/28	Cathepsin D
P45320	51	PKA RII alpha	I41720	130	Integrin beta 1
C12620	40	Crk	K55520	100	KAP3A
B36320	180	Brm	A31320	79	AKAP79
C45520	90	Calnexin	L60620	52	LSP-1
P22520	74	PKC lambda	P27820	130	p130 Cas
N15920	47	NCK	P20120	68	Sam68
S40220	32	Syntaxin 4	D76320	45	DFF45
E27620	25	eIF-4E	G76220	27	GS27
B71520	135/140	TFII-I	B22020	220	BRCA1
G19020	190	p190	D57220	100	Dyrk
C40920	97	CDC27	M77120	56	MST1
M62720	70	MEF2D	N74120	42	Na,K ATPase beta 3
H59520	57	hILP/XIAP	R41220	74	RIP
P73420	40	PKA C	M77320	52	MST3
R26320	28	RhoGDI	H77220	36	Heme Oxygenase 2
N25720	17	Nm23	P88320	37/42	pICln
D28120	165	Desmoglein	I86220	200	Integrin beta 4
M94120	127	MSH3	D90420	119	DBP2
N61020	100	NAT1	65941A	80	Ku80
H53220	70	Hsp70	R86820	58	RIG-G
A29220	55	Acetylcholine Receptor beta	N89620	49-68	NMT-1
E16220	42	ERK2	T96120	180	Topo II beta
G59720	23	GST-pi	556597	100	DNA Topo I
I31220	300	IP3R-3	K95820	75	hcKrox
A59820	149	AKAP149	P33820	87	Phospholipase C delta 1
C19220	92	beta-Catenin	M66720	43	MKP2
C68220	80	CLA-1	L11520	24	LITAF

F23220	46	FPTase beta	14101A		Abl
R64820	36	Ref-1	G64520	110	GPI-Phospholipase D
K25020	27	Kip1/p27	P42820	90	80K-H
D29720	160	DAP Kinase	C50820	50	CDC37
H72320	120	HIF-1 alpha	N79420	26	Nip1
P65620	62	p62 lck ligand	13981A		Raf
F26820	48	FPTase alpha	F56020	72/68	FKBP65
I58620	32	Inhibitor 2	I81720	104	Integrin beta 3
S11120	22	Smac/DIABLO	F51420	51	FKBP51
139320	95/105	Sp1 Pharmingen	C13020	100/79	hCNK1
N48120	238	NuMA	I95020	45	I kappa B epsilon
H44620	110	Hsp110	E12020	180	EGF Receptor
D39920	90	DRBP76	S21520	92	Stat5
T30420	72	TAFII70	P78020	63	PKB Kinase
C45820	63	CDC25B		45	Mek 1
C25820	40	CDC34	M92920	81	Mre11
F46820	29	14-3-3 epsilon	C88020	60	Cyclin A
K72820	395	Ki-67	E46420	30	EB1
V75320	150	Integrin alpha 2/VLA-2	M54020	42/40	Mcl-1
P47120	116	PYK2/CAK beta	T70120	190	Thrombospondin-1
M14020	90	MCM5	N52920	130	iNOS/NOS Type II
N43620	62	Nucleoporin p62	P65920	59	PKB alpha/Akt
G29620	37	G beta	B22620	26	Bcl-x
U66820	18	UbcH7	N32020	130	iNOS/NOS Type II
C50920	135	Contactin	P15820	78	beta PIX
D84920	82	DDX1	A47520	55	beta-Arrestin1
N67620	65	NF-kappa B p65	P63720	140	PRK2
A56920	51	Annexin VII	D27120	100	Dynamin II
C57820	20	Caveolin 2	C14520	50	Csk
M85020	113	MCAM	P23720	33	Pit-1
I87320	87	IKK beta	G95320	58	gp91phox
A84320	55	ALDH	U15420	40	Ufd1L
M85420	37	MKK3b	P79320	160	p160
S11320	22	Smac/DIABLO	C21620	102	alpha-Catenin
D86520	261	DNA Polymerase epsilon cat.	B80520	77	Btk
G85920	94	Glucocorticoid R	P44120	47	Pleckstrin
F88920	74	FBP	U95420	100	UBE3A
S87420	32	SIP1	T94720	72	TIEG2
A15220	21	p21-Arc	C99420	60	Chk2
E35520	90/110	EPLIN	C92720	44	CD40
A91020	160	Adaptin delta	T13820	215	Tensin
S98420	72	Skb1Hs	P16920	180	PDGF Receptor beta
P95220	53	PAF53	R61120	40	Ron alpha
R37920	30	RAP30	R56320	24	Rab11
T81920	135/140	TFII-I	G11420	195	p190-B
G88620	230	p230 trans Golgi	P41620	96	Disabled-2/p96
R90220	73	Reps1	D99320	88	DMPK
E88120	43	ECA39	I10220	50	ILK
T90920	170	Topo II alpha	B84220	528	BRUCE
P63420	100	Plakophilin 2a	W82920	162	WRN
N90120	65	NMT-2	N83220	72	Nurr
H88820	42	hPrp18	N83420	55	No55
L10020	52/75	LEDGF	P10620	38	PEX19
F67720	18-24	basic FGF	S97820	100	SH2-B
G35720	206	GBF1	G93720	84	Golgin-84
M13120	105	MCM6	A78520	70	Aralar
E84420	47	beta-Enolase/ENO-3	G49220	50	GFAP

A99520	121/84	AKAP121/S-AKAP84
C11020	290	Collagen VII alpha 1
H90720	140	hPrp16
L69320	47	La Protein
N17220	220	Nestin
S78120	92	SRPK1
X96820	55	XRCC4
H10520	28	KNP-1/HES1
P92020	400/500	Plectin
P32520	96	PMS2
U10820	95	UBA2
66201A		DP-1
C25020	221	CHD3
F11020	74	FIP-2
H22420	40	Hsp40
R10820	50/46/33	BAG-1
P91720	165	PI3-Kinase C2 beta
N22220	88	Nup88
E11620	112	Exportin-1/CRM1
K91620	70	Ku70
S57320	45	SGT1
R33620	80/100	RanBP3
U24020	146	Ufd2/E4
C78820	43	Cathepsin L
P46020	19	p19Skp1
A63920	280	ABP-280
N30020	140	eNOS/NOS Type III
E50020	71	EFP
L85220	56	LXR
J31920	39	Jun
K38420	140	Kalinin B1
S21220	113	Stat2
I29320	48	ISGF3 gamma
F37720	37	Fas Ligand
E41120	180	EEA1
P16520	82	PKC alpha
L64920	63	LSF
P75720	32	PITP alpha
F14220	18–24	basic FGF
P41920	150	p150Glued
D25520	100	Dynamin
P67320	50	Pax-5
A13920	38	Annexin I
C26120	130	Cadherin-5
P54320	68	Paxillin
I58220	38	I kappa B alpha
B61220	26	Bcl-x
C24420	21	Cip1/WAF1
N38620	140	eNOS/NOS Type III
R14320	68	A-Raf
A29920	36	Annexin IV
C43420	22	Caveolin 1
B92320	137	53BP2
A43920	112	Adaptin alpha
H46720	75	HS1
E51120	46	E2F1
R22020	21	Rap1
B97920	23	Bid
C10020	90-115	CD54/ICAM-1
P10020	143	PEX1
D10520	27	drp1
V11220	11	V-1/myotrophin
E14720	43/21	p43/EMAP II precursor
M16520	190	MNK
B13020	82	BERP
P14120	220	Pericentrin
X97720	182	Xin
E10020	100	eEF-2 Kinase
N10020	56	Nek3
680405		Caspase-6
A98020	175	Attractin
R19120	74	c-Raf-1
S88720	40	Syntaxin 11
S30520	11	S100P
A82620	120	APM
Z91420	74	ZBP-89
K82520	53	Kanadaptin
F10520	17	FHIT
N87720	104	Neuropilin-2
P87520	60	PTRF
P35820	44	Phosphatase Methylesterase-1
H11020	10	Hsp10
S91320	150	SHIP-1/p150Ship
S85320	130	SPA-1
F89320	50	FEN-1
C15720	105	Chromogranin B
S94220	80	Synapsin I
S10320	52	Syndapin I
S34220	36	SCAMP1
G22020	22	Guanylate Kinase
C26520	80	Cypher1
M89520	22	MKBP
Labc20		LFB3
C36820	51	Carbonic Anhydrase XIV
F20120	135/100	4.1N
B11520	40	Bub3
H99020	60	Hsp60
S92220	27	Syntaxin 8
E17520	110	espin
M16020	35	Metaxin
A12520	105	AIP1
P97220	58	PKR
N13320	30	NES1
A94620	540	Apolipoprotein B-100
N91220	110	NABC1
K95120	80	Katanin p80
R97120	110	RECK
K32120	34	KAP
I96720	40/44	IGFBP-3
R79520	125	p116Rip
J24320	130	JAK1
C20820	120	E-Cadherin
C43820	180	Clathrin Heavy Chain
M76120	120	Mint2

C47620	43/28	Cathepsin D	O84720	46	3-Oct
I53820	195	IQGAP1	S29820	10	S100L
G65120	130	GM130	P17920	120	pp120
H80920	50	HDAC3	Z61220	51	ZRP-1/TRIP6
A92820	130	Apaf-1	A12820	30	Acrp30/Adiponectin
M60920	100	MCC	D30120	123	DLC-1
F58420	79	5-Lipoxygenase	C21120	86	Chromogranin A
V40620	46	VASP	G13520	24	GAGE
T87920	32	TRP32	C97020	36	Caspase-14
E83020	50-53	EBP50	N74320	60	NKT
E83920	120	Eg5	N41520	155	nNOS
S12820	92	Stat5A	N12520	92	NHE-1
S84820	38	SII/TFIIS	Z45420	83	Zyxin
U61320	18	Ubc9	D10020	41	Doublecortin
S90020	72	SNX2	N12920	80	NHE-3
R90820	60	hRAD9	C16420	55	Calsequestrin
J36020	38	JAM-1	C36220	22	Caveolin 1
V94820	27	Vti1b	L13020	72	LIMK1
Jxxx20		Jagged 2	E93220	46	mEPHX
T20220	135	tyk2	R32820	90	Rin1
E90320	95	Endoglin	I68120	48	IGTP
R35420	45	RCC1	L37820	65/74	Lamin A/C
V84120	110/220	VAP-1	C45220	145	CAPS
N10320	150	tNASP	F83520	70	Fnk
S81220	95	Sin	P42220	40	Prostasin
P98520	130	Phospholipase C beta 4	G10920	67	GAD67
F91120	95	FXR2	C38920	33	CENP-H
E87220	50	Endothelin 1 Receptor	C11320	45	Casein Kinase II alpha
C95920	150	Ceruloplasmin	A10020	46	ASS
L16120	110	LDLB	I89920	48	IKK gamma
A99120	82	AKAP82	T31820	150	TNIK
P96520	47	PTEN	H83120	45	HsORC4
C41720	60	Calreticulin	C34120	62	Collybistin
P39820	41	PBK	S10120	48	SQS
V39420	33	VAP33	T33920	135	TAFII135
E78320	130	erg2	M72220	50	MEK5
P76020	97	PPEF-2 long form	T97620	47	Thrombin Receptor/PAR1
C80420	46	CD38	L89820	53	LCB1
G96620	16	GRIFIN	P54720	53	PKA RII beta
R54520	180	ROCK-II/ROK alpha	K34220	200	KIF1A
P57920	120	PRK1	L74520	53	LAP2
S21320	92	Stat3	C82320	54	Chk1
C39520	66	CoRest	M91620	54	MARCO
S71420	54	SRP54	H35920	120	Hip1R
R20620	36	RACK1	Z37520	51	ZPR1
R66320	24	Rab8	T36120	120	TIP120
C27220	180–220	CD45	B35220	61	Beclin
F71620	130	FYB/SLAP-130	K35620	110	KIF2
K48020	97	Karyopherin beta	H65520	55	HSF4
P54420	72	PTP1D/SHP2	P15320	39	PECI
L15620	56	lck	P33420	58	Prenylcysteine Lyase
P56720	36	PCNA	C32820	48	CtBP1
V47020	20	VHR	D31520	56	Dok2/p56 dok2
R20720	250	RPTP beta	G34320	55	GRASP55
L30220	200	L1	N10720	95	NBS1
L76620	110	Lamp-1	Z34720	52	ZIP Kinase
B63620	80	BMX	N62820	22	Ninjurin

R43320	48	RBBP
A40720	36	apoE
R73920	21	Rho
D73020	125	DNA Polymerase delta
C42920	100	CAS
M36820	78	Moesin
F19720	59	fyn
E35820	42	Flotillin-2/ESA
D58520	21	DHFR
M75820	200-220	MAP4
R27020	130	Rb2
A35620	106	Adaptin beta
P15120	79	PKC theta
C23420	62	Cyclin B
N52120	46	Nek2
H57520	24	hsMAD2
N93320	320	C-Nap1
T73620	160	TIF2
R68320	110	Rb
T10120	70	TAP
C67520	53	CART1
C31720	32	Caspase-3/CPP32
G72420	15	GS15
D64220	350	DNA-PKcs/p350
Z72720	220	ZO-1
C39220	140	CTCF
K57620	105	KRIP-1
T37220	85	Transferrin Receptor
E72920	69	ERp72
I75620	48	Caspase-2/ICH-1L
S55420	31	Syntaxin 6
N34920	72	NSP1
T27720	140	Tat-SF1
S31620	33	Synaptophysin
N41320	95	Neurotensin Receptor 3
Z10420	67	ZFP-37
N32720	56	Ntk
67391A		
554090		Cdk5
67381A		Bik
662305		Caspase 8
556452		Ercc-1
554286		JNK1
556367		CD3 zeta
15811A		c-erb-B2
556596		14-3-3
556453		XPA
14821C		Cyclin D2
P11120		Phosphotyrosine-PY20
P39020		Phosphotyrosine-PY69
S99220	100	Phospho-Stat6 (Y641)
C91520	22	Phospho-Caveolin (Y14)
S89120	100	Stat6
C13620	22	Caveolin 1
F25020	125	Phospho-FAK (Y397)
I33220	48	IRF1
F15020	125	FAK
B36420	23	Bad
E12120	180	EGF Receptor (activated form)
550747	59	Phospho-Akt
P23520	68	Phospho-Paxillin (Y118)
P49620	68	Paxillin
S12220	92	Phospho-Stat5 (Y694)
P21020	53	p53
M12320	44/42	ERK1

Fold Change = semiquantitative value representing general trend of protein changes for the experimental sample relative to control
"-" = represents a decrease in protein level in the experimental sample relative to control
"+" = represents an increase in protein level in the experimental sample relative to control
Confidence level = protein changes are listed in order of confidence, 5 being the highest confidence
a) Changes greater than 2 fold in triplicate from good quality signals are of highest confidence (level 5)
b) Followed by changes 1.5-1.9 fold in triplicate from good quality signals (level 4)
c) Changes 1.25-1.5 fold in triplicate (level 3)
d) Changes in triplicate from low signals are listed next (level 2)
e) And finally changes greater than 2 fold in duplicate from good quality signals (level 1)
div/0 = represents presence verses absence of a protein, fold change calculation yields irrational number
(PF): = putative function
Highlighted proteins are those in which a change was visually observed in triplicate
Note: Data for pleiotropic (multifunctional) proteins may be replicated under separate functional headings

Proteins by Function, correlated with

A2058 Protein Expression Fold Changes Compared to Control, 24 hours post exposure to 532 nm light

Protein ID	Confidence Level	Change +/-	Avg. Fold Change	Fold Change Run 1	Fold Change Run 2	Fold Change Run 3	References, Brief Functionality
Protein Destruction, and protein-conjugating enzymes which mark proteins for destruction							
Ubc9	5	div/0	7.45	7.45	div/0	div/0	(Nuber, 1996) (NCBI, P51966). Ubiquitin-conjugation
UbcH7	5	+	#DIV/0!	**div/0**	**div/0**	**div/0**	(Nuber, 1996). (NCBI, P51966). Ubiquitin-conjugation
UbcH6	1	+	2.17		div/0	2.17	(Nuber, 1996). (NCBI, P51966). Ubiquitin-conjugation
CUL-3-79	3	+	1.70	1.34	1.68	2.09	(Singer, 1999) S-phase control, CUL-3 targets cyclin E for destruction
GST-pi	4	-	3.92	**3.41**	**6.67**	**1.69**	(Kano, 1987) (Ali-Osman, 1997) (Zhou, 1997) S-glutathione conjugation
SNX1	4	-	4.96	10.97	1.59	2.32	(NCBI NP_003090)(Haft, 1998) Binds epidermal growth factor receptor
p47A-44	2	+	1.47	1.67	1.47	1.28	(Dell'Angelica, 1997) (Hirst, 1998) endosomal protein sorting to lysosome
Prenylcysteine Ly	2	+	1.79	1.27	2.51	1.59	(NCBI NP_057381)(Tschantz, 1999) lysosomal prenylcysteine degradation
Mitotic regulation: signal protein production, phosphorylation or destruction; req'd substrates							
Ubc9	5	+	7.45	7.45	div/0	div/0	(Nuber, 1996) (NCBI, P51966) Ubiquitin-conjugation, destruct. of key mitotic regulators
UbcH7	5	+	#DIV/0!	**div/0**	**div/0**	**div/0**	(Nuber, 1996) (NCBI, P51966) Ubiquitin-conjugation, destruct. of key mitotic regulators
UbcH6	1	+	2.17		div/0	2.17	(Nuber, 1996) (NCBI, P51966) Ubiquitin-conjugation, destruct. of key mitotic regulators
Sam68-65	5	-	6.50	**4.02**	**10.86**	**4.60**	(Richard, 1995) (Lee, 1999) adaptor protein, promotes mitosis
SHC-46	4	-	2.16	2.98	1.91	1.59	(Pelicci, 1992)(Chow, 1998) early growth factor signal intermediates

Mitotic regulation: signal protein production, phosphorylation or destruction; req'd substrates (Cont.)							
ShcC-73	**4**	-	2.65	**2.22**	**3.97**	**1.76**	(O'Bryan, 1996) epidermal factor growth receptor signalling cascade
SHC-67	3	-	1.69	1.87	1.69	1.49	(NCBI NP_003020) (Pelicci, 1992) mediates signals for growth, metabol.
Csk	2	+	1.52	1.70	1.58	1.28	(NCBI P41240)(Brauninger, 1993) negative regulator of Src family kinases
A-Raf	5	-	3.13	**2.10**	**4.57**	**2.72**	(Lee, 1994)(Lee, 1996) proto-onco gene, cell growth and development
RanBP1-27	5	-	3.79	5.26	3.87	2.23	(NCBI, P43487) (Hayashi, 1995) Stabilizes GTP-bound state of Ran
RanBP1-32	3	-	11.48	2.12	30.91	1.41	(NCBI, P43487) (Hayashi, 1995) Stabilizes GTP-bound state of Ran
Ran	1	+	6.03	2.14	9.92		(NCBI, P43487) (Hayashi, 1995)(Ren, 1993) DNA synth. & mitotic initiation
p160	**5**	-	2.27	**2.24**	**div/0**	**2.30**	(Favier, 1994) cell proliferation and differentiation, c-Myb target gene activat.
Cdk1/Cdc2	4	-	1.87	1.82	1.54	2.26	(NCBI P06493)(Draetta, 1988)(Sherr, 1995) cyclin-Cdk induce mitotic entry
Cdk2	3	-	1.99	1.86	1.48	2.63	(NCBI XP_049150)(Gu, 1992) G2, S phase cyclin-dependent cell cycle reg.
KAP	3	+	2.81	1.28	5.38	1.77	(NCBI Q16667)(Hannon, 1994) CdK associated phosphatase
pan ERK-39	4	-	1.79	1.84	1.71	1.83	(Sivaraman, 1997)(Cobb, 1995)(aka MAPK, mitogen-activated (P) kinases)
Casein Kinase I e	3	-	2.82	4.99	2.00	1.47	(NCBI P49674) (Fish, 1995) regulation of DNA replication and repair
SNX1	4	-	4.96	10.97	1.59	2.32	(NCBI NP_003090)(Haft, 1998) Binds epidermal growth factor receptor
CUL-3-79?	3	+	1.70	1.34	1.68	2.09	(Singer, 1999) S-phase control - CUL-3 targets cyclin E for destruction
DHFR	3	-	2.21	1.48	1.42	3.72	(NCBI P00374)(Wells, 1996) synthesis of thymidine, amino acids, purines
GSK-3 beta	3	-	2.41	3.09	1.32	2.83	(NCBI P49841)(Stambolic, 1994) phosphorylates mult. regulatory proteins
No55-57	3	-	3.10	1.40	4.20	3.70	(NCBI Q92791)(Ochs, 1996) localized to chromosome surface during mitosis
No55-54	2	-	3.78	1.97	2.07	7.31	(NCBI Q92791)(Ochs, 1996) localized to chromosome surface during mitosis
Caveolin 1	2	-	1.79	1.68	1.67	2.01	(NCBI NP_001744)(Wary, 1998)(Conrad, 1995) G-protein signalling
Glucocorticoid R	2	+	1.37	1.49	1.25	1.36	(NCBI P04150)(Leclerc, 1991)(Reichardt, 1998) transactivation/repression
p70s6k	2	+	2.07	1.36	3.30	1.57	(NCBI P23443) (Grove, 1991) (Romanelli, 1999) mitogenically stimulated
PYK2/CAK beta-	2	+	2.22	2.69	1.90	2.07	(NCBI Q14289)(Lev, 1995) links G proteins to MAP kinase pathway
Ras-GAP	2	+	3.74	4.88	4.06	2.27	(NCBI P20936)(Scheffzek, 1996) negative regulator of P21ras
MST3-52	2	-	5.30	2.10	10.68	3.11	(NCBI Q9Y6E0)(Zhou, 2000) activates p42/44 mitogen-activated protein kinase
PI3-Kinase-76	1	+	2.98	3.38	2.58		(Kapeller, 1994) sig. transduction phophorylates signaling molecule phosphatidylinositol
p190	1	-	#DIV/0!	div/0	div/0		(NCBI NP_001164)(Burbelo, 1995) Ras-GAP associated GTPase activator
p19Skp1	1	+	7.35		7.35	div/0	(NCBI P34991) (Zhang, 1995) in S phase complexes w/ Cyclin A and Cdk2
Rap2	1	+	3.42	3.42	div/0		(Ohba, 2001)(PF): suppresses Ras-mediated activation of ERK/MAP kinase cascade
Apoptosis							
LITAF	4	-	2.12	1.85	1.53	2.97	(NCBI NP_004853)(Myokai,1999); TNF-a link to p53?
alpha-Synuclein	4	+	1.53	div/0	1.53	div/0	(NCBI P37840) (Hashimoto, 2002) inactivation of jun kinase stress signaling pathway
BAG-1-37	3	-	1.71	1.35	2.21	1.58	(NCBI Q99933) anti-apoptotic activity; heat shock protein (Hsp70) reg.
hILP/XIAP	3	+	6.63	1.27	1.42	17.20	(NCBI P98170)(Duckett, 1996) inhibitor of caspase 3, 7 induced apopto.
TIAR-43	3	-	1.68	1.70	1.26	2.08	(NCBI A46174)(Kawakami, 1992)(Taupin, 1995) Fas-mediated apoptosis

Apoptosis (Cont.)							
MST-1	3	+	1.74	2.44	1.51	1.26	(NCBI Q13043)(Taylor, 1996) Ser/Thr kinase, apoptosis signal feedback amplification
Apaf-1	2	+	1.57	1.33	2.12	1.28	(NCBI O14727)(Zou 1997)(Cain, 1999) caspase-9 activation
Fas/CD95/APO-1	2	-	2.52	3.02	2.65	1.90	(NCBI P25445)(Itoh, 1991)(Hanabuchi, 1994) Apoptotic signal molecule
JNKK1/MKK4	2	-	1.71	1.56	1.27	2.30	(NCBI P45985)(Lin, 1995)(Derijard, 1995) phosphorylates JNK & p38
p43/EMAP II prec	1	-	5.36		7.74	2.97	(Knies, 1998) EMAP II recruits PMNs to phagocytize aopoptotic cells
Peroxiredoxin V	1	+	#DIV/0!	div/0		div/0	(NCBI NP_036226)(Knoops, 1999)(Zhou, 2000) overexpression inhibits apoptosis
Smac/DIABLO	1	-	#DIV/0!		div/0	div/0	(NCBI NP_063940)(Verhagen, 2000) antagonizes XIAP, if cytosolic
Transcription							
La Protein	**4**	**-**	1.71	**1.63**	**1.81**	**1.69**	(Fan, 1997) (Gottlieb, 1989) (NCBI P05455) protects nascent transcripts
FACTp140-126	3	-	2.01	3.24	1.30	1.48	(NCBI AAD43978) (Orphanides, 1999) reg. access to DNA in chromatin
TAF-172	3	-	1.79	2.39	1.56	1.43	(NCBI O14981)(Chicca, 1998) with TBPs promotes assembly of RNA polymerases I-III
TLS	3	-	1.75	1.39	1.66	2.22	(NCBI AAB27102)(Crozat, 1993) nuclear receptor, gene transactivation
CHD3-384	3	-	3.18	1.46	4.92	3.16	(NCBI AAB87383) (Ogas, 1999) nucleosome remodelling transcription repressor
Glucocorticoid R	2	+	1.37	1.49	1.25	1.36	(NCBI P04150)(Leclerc, 1991)(Reichardt, 1998) transactivation/repression
p54nrb-56	2	-	2.57	1.37	3.34	2.99	(NCBI Q15233)(Dong, 1993) premCCCRNA splicing, DNA transcription factor
p54nrb-52	1	-	3.25	3.27		3.23	(NCBI Q15233)(Straub, 1998)(Basu, 1997) activates topoisomerase I
CtBP1-41	1	+	2.90		2.57	3.24	(NCBI Q13363) (Sewalt, 1999) corepressor for transcriptional factors
Translation							
eIF-5a	**5**	**+**	7.57	**12.54**	**div/0**	**2.60**	(Weaver, 1999)(Hofmann, 2001) translation. initiation, nuclear transport function
TRAX	3	-	1.76	1.77	1.42	2.11	(NCBI NP_005990)(Aoki, 1997) regulation of RNA transport/translation
p54nrb-56	2	-	2.57	1.37	3.34	2.99	(NCBI Q15233)(Dong, 1993) preMRNA splicing, DNA transcription factor
p70s6k	2	+	2.07	1.36	3.30	1.57	(NCBI P23443) (Grove, 1991) phosphorylates ribosomal protein S6
AKAP149-183	1	+	3.55	3.31	3.80		(Trendelenburg, 1996) phosphorylation-dependent regulation of RNA-processing
p43/EMAP II prec	1	-	5.36		7.74	2.97	(Norcum, 2000)(Kao, 1994) P43 complexes w/ aminoacyl-tRNA synthetase
Vessicular Trafficking							
Neurotensin rece	4	-	2.01	1.68	2.16	2.19	(NCBI NP_002950) neuropeptide: dopaminergic transmission; sortilin
ARF-3	3	+	1.37	div/0	1.37	div/0	(NCBI NP_001649) vesicular trafficking, vesicle formation & fusion
BiP/GRP78	3	+	3.05	1.32	div/0	4.79	(NCBI P11021) (Linnick, 1998) ER chaperone for malfolded or unassembled proteins
GS27	3	+	2.75	1.46	4.59	2.19	(NCBI O14653) (Lowe, 1997) medial-to-trans-Golgi protein movement
Caveolin 1	2	-	1.79	1.68	1.67	2.01	(NCBI NP_001744)(Wary, 1998)(Conrad, 1995) lipid transport
Complexin 2	2	+	#DIV/0!	div/0	div/0	div/0	(NCBI O42105)(McMahon, 1995) exocytotic vescicle fusion regulation
p47A-44	2	+	1.47	1.67	1.47	1.28	(Dell'Angelica, 1997) (Hirst, 1998) endosomal protein sorting to lysosome
Rab11	2	-	1.51	1.57	1.61	1.36	(NCBI CAA40064)(Ullrich, 1996) pericentriolar recycling compartment
Rab8	2	-	3.25	1.42	2.46	5.86	(NCBI CAA40065)(Peranen, 1996) TGN to basolateral plasma membrane
Dystrobrevin-84	2	+	2.08	1.37	3.49	1.38	(NCBI Q9Y4J8) (Blake, 1996) neuromuscular synapse formation and maintenance
Bet1	1	+	3.73	3.73	div/0		(NCBI NP_005859)(Hay, 1996) vesicular transport from (ER) to the Golgi

Cell Adhesion / Motility / Microtuble Function							
Ninjurin-18	4	+	1.78	**1.64**	**1.91**	**1.79**	(NCBI NP_004139) (Araki, 1996) Nerve injury-induced; mediates homophilic adhesion
RECK	3	+	1.76	1.98	1.84	1.46	(NCBI BAA34060) (Takahashi, 1998) inhibits tumor invasion/metastasis
ROCK-I/ROK bet	3	-	2.82	3.32	3.79	1.36	(Ishizaki, 1996)(Tominaga, 1998) Rho-kinase: regulation of focal adhesion
Ral A	3	-	1.70	1.38	1.92	1.81	(NCBI TVHUAA) (Polakis, 1989) (Gildea, 2002) growth-factor activated motility
Gephyrin	3	+	1.75	1.49	1.51	2.26	(NCBI NP_065857) (Prior, 1992) anchors the GlyR to subsynaptic microtubules
Contactin	2	-	4.88	1.28	2.56	10.80	(NCBI NP_001834)(Berglund, 1994) neuronal cell adhesion protein
Dematin-61	**3**	**-**	1.50	**1.42**	**1.60**	**1.48**	(NCBI I39062) (Azim, 1995) erythroid actin bundling protein
Dematin-57	2	-	2.36	3.07	2.60	1.40	(NCBI I39062) (Azim, 1995) erythroid actin bundling protein
FAK	2	+	1.99	1.32	2.04	2.62	(NCBI Q05397) (Hildebrand, 1993) Focal Adhesion Kinase, adhesion sig.
Integrin beta 1	1	-	#DIV/0!	div/0	div/0		(Balzac, 1994) (NCBI P05556) mediates cell-cell & cell-matrix adhesion
GelsolinG37820	1	-	16.05	30.01	2.09		(NCBI 1211330A) (Kwiatkowski, 1986) severs actin filaments
Stathmin/Metabla	1	+	#DIV/0!		div/0	div/0	(NCBI P16949)(Brattsand, 1994) microtubule disassembly
Immunology							
CD54/ICAM-1-9	4	+	1.73	1.54	1.87	1.79	(NCBI P05362) LFA-1/Integrin ligand; Rhinovirus receptor
Integrin beta 1	1	-	#DIV/0!	div/0	div/0		(NCBI P05556) (Greve, 1989) leukocyte-epithelial cell adhesion
GAGE	4	+	1.89	2.39	1.66	1.61	(NCBI AAA82744) (Van den Eynde, 1995) Melanoma surface antigen
LITAF	4	-	2.12	1.85	1.53	2.97	(NCBI NP_004853)(Myokai, 1999) LPS-induced TNF-a production
tyk2	3	+	1.51	1.38	1.62	1.55	(NCBI TVHUY2)(Velazquez, 1992)(Watling, 1993) IFN a/b response transcription
Fas/CD95/APO-1	2	-	2.52	3.02	2.65	1.90	(NCBI P25445)(Itoh, 1991)(Hanabuchi, 1994) Apoptotic signal molecule
DNA Repair							
MSH6	**3**	**-**	2.57	**3.50**	**1.46**	**2.75**	(NCBI P52701)(Gradia, 1997) G/T mismatch, insertion/deletion loop repair
XPA	1	-	2.95		3.72	2.19	(NCBI JG0190)(Aboussekhra, 1995)(Evans, 1997) excision repair
Rac1-21	1	+	#DIV/0!	div/0		div/0	(NCBI TVHUC)(Cho, 2002) DNA repair via Ras/PI3K/Rac1/NADPH oxidase-dep. Path
Miscellanous Functions							
BPntase	3	-	1.56	1.54	1.89	1.25	(NCBI NP_006076) "bisphophate 3'-Nucleotidease"; salt, sulfur, Li+ toxicity
HAP1-102	3	-	1.56	1.27	1.58	1.84	(NCBI S67493)(Li, 1995). Huntington disease-associated protein
NMT-2	3	-	1.54	1.68	1.50	1.45	(Giang, 1998) N-myristolyltransferase, cotranslational mod. of signalling protein
mEPHX	2	-	1.56	1.84	1.46	1.38	(NCBI A29939)(Hassett, 1994) reactive epoxide metabolism
Acrp30/Adiponec	1	+	2.79	2.36	3.21		(Scherer, 1995)(Bogan, 1999) adipocyte secreted, metabolic homeostatic factor
Na,K ATPase bet	1	-	3.33		4.13	2.52	(NCBI AAB61713) (Stengelin, 1997) Na+ and K+ ion pumping protein
p62 lck ligand-59	1	+	4.46	5.77		3.15	(Puls, 1997)(Joung, 1996)(Foger, 2000) cytoplasmic, lck which is associated w/ CD44
Function Unknown							
drp1	**5**	**-**	**2.68**	**2.02**	**3.11**	**2.91**	(Deyo, 1998) density-regulated protein, expression increased at high cell density